



Molecular markers of pre-natal non-scorbutic vitamin C deficiency in foetal and young guinea pig brains

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MOLECULAR MARKERS OF PRE-NATAL NON-SCORBUTIC VITAMIN C DEFICIENCY IN FOETAL AND YOUNG GUINEA PIG BRAINS

PhD Thesis 2014 • Maya Devi Paidi



Molecular markers of pre-natal non-scorbutic vitamin C deficiency in foetal and young guinea pig brains

PhD Thesis 2014
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Preface

The work for the current PhD thesis was performed from 01/09/2009 - 09/01/2014 (with an interruption of 14 months on maternity leave) at the section of Experimental Animal Models, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen. The research was funded by the Danish Research Councils, University of Copenhagen and the LIFEPHARM Centre for In Vivo Pharmacology.

In this thesis chapter 1 is a background on vitamin C, chapter 2 describes vitamin C transport and chapter 3 describes the effects of vitamin C in the brain followed by discussion and conclusion before the manuscripts are presented.

This thesis is in part based on the following manuscripts referred to in the text by their Roman numerals

Manuscript I: Increased expression of vitamin C transporters in liver but not kidney and brain during deficiency

Manuscript II: Chronic vitamin C deficiency promotes redox imbalance in the brain but does not alter sodium-dependent vitamin C transporter 2 expression

Manuscript III: Prenatal vitamin C deficiency results in differential levels of oxidative stress during late gestation in foetal guinea pig brains

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Abbreviations

VitC	vitamin C
AR	ascorbyl radical
DHA	dehydroascorbate
SOD	superoxide dismutase
GSH	glutathione
CAT	catalase
GPx	glutathione peroxidase
NT	nitrotyrosine
LPO	lipid peroxidation
MDA	malondialdehyde
HNE	hydroxynonenal
NMDAR	N-methyl-D-aspartate receptor
SVCT	sodium dependent vitamin C transporter
GULO	gulonolactone oxidase
CNS	central nervous system
GD	gestational day
DOPA	dihydroxyphenylalanine
DOPAC	dihydroxyphenylacetic acid
3MT	3methoxytyramine
HVA	homovanillic acid
mEPSCs	miniature excitatory postsynaptic currents
$\bullet\text{O}_2^-$	superoxide
H_2O_2	hydrogen peroxide
$\bullet\text{OH}$	hydroxyl radical
$\text{NO}\bullet$	nitric oxide
ONOO^-	peroxynitrite

Sammendrag

Vitamin C (VitC) er nødvendigt for flere fysiologiske funktioner og er centralt for normal udvikling af hjernen. I dette projekt har vi vist, at hjernen opretholder et VitC niveau på omkring 30 % af kontrolniveauet, mens andre organer udsættes for svær depletering under moderat og absolut mangel på VitC i kosten. Rationen mellem VitC i plasma og cerebrospinalvæske var ens for alle fodringsgrupper. Den selektive tilbageholdelse af VitC i hjernen ved moderat og absolut mangel skyldtes ikke en overordnet regulering af SVCT2 ekspression. Det samme var tilfældet i de unge hjerner fra præ-/postnatal studiet, hvor der desuden blev påvist en øget andel af oxideret VitC og et højere niveau af lipid peroxidering ved VitC mangel. Fundene fra prænatal studiet viste, at føtale VitC niveauer var afhængige af det maternelle VitC indtag og at behovet for VitC i den føtale hjerne falder tæt på drægtighedens afslutning. Siden hjernens behov for VitC ikke var opfyldt under mangeltilstand er forøget superoxid dismutase (SOD) aktivitet sandsynligvis en beskyttende mekanisme. På trods af SOD kompensering var niveauet af lipid peroxidering øget i den føtale hjerne på begge undersøgte tidspunkter i den sene drægtighed. Selvom føtale hjerner med VitC mangel havde oxidativt stress, blev der ikke fundet signifikante forskelle mellem kontrolhjerner og hjerner med mangeltilstand for supplerende markører for oxidativ skade (hydroxynonenal- eller nitrotyrosin modificerede proteiner) eller for apoptose markøren caspase-3. I stedet blev der påvist forskel i ekspression for de målte markører mellem de undersøgte tidspunkter i den sene drægtighed. Samlet set viste vores data, at både hjernen under udvikling og den unge hjerne hos marsvin er prædisponeret for oxidativt stress som følge af subklinisk VitC mangel.

Vores fund kunne have klinisk betydning hos mennesker, idet lavt maternelt indtag af VitC under graviditet kunne resultere i redox ubalance i den føtale hjerne og dermed muligvis have negative konsekvenser for hjernens udvikling hos børn af mødre med VitC mangel. Yderligere giver vores fund indsigt i betydningen af VitC for mindske af oxidativt stress medieret neurodegeneration.

Summary

Vitamin C (VitC) is required for several physiological functions and is pivotal for normal development of the brain. In the current project we have shown that the brain sequesters about 30% of control VitC levels irrespective of differences in the VitC content in the diet while the other organs undergo severe depletion during deficiency and depletion. Ratio of VitC levels from plasma to cerebrospinal fluid (CSF) were the same in all dietary groups. This selective retention of VitC in the brain during deficiency or depletion was not by the mechanism of an overall regulation of SVCT2 expression. This data was supported by a similar finding in the pre/post natal VitC deficient brain which were shown to have increased percentage of oxidized VitC and lipid peroxidation levels. Our findings from low maternal VitC intake showed that foetal VitC levels are dependent on the maternal intake and that the demand for VitC in the foetal brain is higher at an earlier stage of the late gestation. Lack of meeting this demand of high VitC in the deficient brain displayed elevated superoxide dismutase (SOD) activity as a protective mechanism. However, lipid peroxidation levels were increased despite the compensation by SOD in the foetal brain at both investigated time points in late gestation. Although the VitC deficient foetal brains showed oxidative stress, none of the additional oxidative damage markers (hydroxynonenal or nitrotyrosine modified proteins) or the apoptotic marker (caspase-3) showed significant differences between VitC control and deficient brains. Instead, differential expression of the measured markers was detected during the late gestation. Our current data shows that both the developing brain and young brain of the guinea pig is predisposed to oxidative stress due to non-scorbutic VitC deficiency.

We speculate that our findings may have clinical implications in humans due to low maternal VitC intake during pregnancy that may result in redox imbalance in the developing foetal brain or alter brain function in children born to VitC deficient mothers. In addition our findings give insight to the role of VitC in ameliorating oxidative stress mediated neurodegeneration.

Introduction

Vitamin C (VitC) is an important micronutrient required for several important physiological functions [Mandl et al., 2009]. Prolonged low plasma VitC that may result in scurvy was once a devastating illness due to lesser access to fruits and vegetables [Harrison et al., 2010a]. Although reports on scurvy are rare in the western world marginal VitC deficiency ($< 23\mu\text{M}$ in plasma) affects substantial population even in the developed parts of the world [Villalpando et al., 2003]. However, the effects of marginal VitC deficiency are unclear.

VitC in the brain is considered one of the major antioxidants and was proposed to be vital for proper brain development. Brain VitC is several times greater than the plasma and most other organs and especially during deficiency brain is exceptional in maintaining high VitC concentration [Hughes et al., 1971]. Additionally, VitC repletion in the brain is rapid relative to repletion in the plasma suggesting a crucial requirement of high VitC levels in the brain [Tveden-Nyborg et al., 2012]. This finding indicates that the brain preferentially retains VitC. High levels of VitC in the brain are primarily achieved by the sodium dependent vitamin C transporter 2 (SVCT2) [Sotiriou et al., 2002], that transports VitC from the choroid plexus into the cerebrospinal fluid (CSF), from where VitC enters into cells of the central nervous system (CNS) [Rice, 2000]. Lack of SVCT2 results in undetectable VitC levels in the brain leading to death soon/immediately after birth [Sotiriou et al., 2002]. These results emphasize the vital role of SVCT2 in transporting VitC in the brain. *In vitro* studies have suggested an increased expression of the transporter, following decrease of intracellular VitC levels, to likely increase VitC uptake [Savini et al., 2007a; Savini et al., 2007b]. These findings suggest that the selective retention of high VitC in the brain during deficiency could be due to an adaptive mechanism of SVCT2 up-regulation to increase VitC uptake, leading to the hypothesis 1. Hence in the Manuscript I, the regulation of SVCT2 expression was investigated following VitC deficiency as well as depletion, together with analysis of VitC transport from plasma to the CSF.

It has been shown previously that high levels of brain VitC are essential to provide protection from oxidative stress [Harrison et al., 2010a; Harrison et al., 2008]. Hence, increased VitC transport to the brain by an up-regulation of SVCT2 as suggested by *in vitro* studies [Savini et al., 2007b] may not alleviate oxidative stress in the brain during VitC deficiency, leading to the hypothesis 2. In our earlier study we have shown that prenatal non-scorbutic VitC deficiency results in hippocampal

volume reduction in the postnatal brain which was not improved despite repletion to control levels of VitC after birth [Tveden-Nyborg et al., 2012] suggesting that pre-natal VitC deficiency affects the brain and that the effect may be irreversible. Therefore we tested hypothesis 2 in Manuscript II by investigating the postnatal effects of pre-natal VitC deficiency, on oxidative stress and SVCT2 expression in the brain. We also assessed if postnatal repletion of VitC could reverse the effects of VitC deficiency in the brain.

An earlier study by our group has shown that non-scorbutic VitC deficiency in the early postnatal life results in a 30% reduction in hippocampal neurons and impaired spatial memory [Tveden-Nyborg et al., 2009]. In another study we have reported that cellular migration into the dentate gyrus granular layer of hippocampus was reduced in the prenatally deficient animals associating hippocampal impairment as an effect of VitC deficiency [Tveden-Nyborg et al., 2012]. These findings showed the effect of VitC deficiency in the brain at an early postnatal time or later and suggests that VitC deficiency due to low maternal intake of VitC may also affect the pre-natal brain during gestation. The foetal brain is especially vulnerable due to rapid growth and immature antioxidant defence mechanisms [Lykkesfeldt et al., 2007]. Others have previously suggested that the foetal brain requires an increase in VitC with progression in brain development [Harrison et al., 2010b; Zalani et al., 1987]. The inability of meeting the requirement of high VitC due to maternal VitC deficiency imposes the foetus to oxidative stress leading to hypothesis 3. Hence we investigated the effect of pre-natal VitC deficiency on oxidative stress in the foetal brains at two gestational time points and the findings are presented in the manuscript III.

Guinea pig is the animal model of our interest to investigate the effects of VitC deficiency because of its evolutionary loss of the functional gulono-g-lactone oxidase (*Gulo*) gene. As a result they cannot synthesize VitC endogenously and depend on the dietary intake similar to human beings [Nishikimi et al., 1992]. Moreover, guinea pig foetus has an extensive brain development during gestation, with the occurrence of brain growth spurt nearly two weeks before birth [Dobbing and Sands, 1970]. Also, we have previously established that 100mg/Kg diet in guinea pigs leads to a non-scorbutic VitC deficiency [Tveden-Nyborg et al., 2012]. From the current work we expected to understand the role of Svct2 in sequestration of the brain VitC and the effect of non-scorbutic VitC deficiency on oxidative stress in the postnatal and prenatal brain.

PhD hypotheses

Hypothesis 1: High brain VitC relative to other organs is maintained in guinea pigs during deficiency through induction of sodium dependent vitamin C transporter, SVCT2.

Hypothesis 2: Despite its preferential retention, non-scorbutic VitC deficiency increases oxidative stress in the brain of guinea pigs.

Hypothesis 3: The guinea pig foetus is not protected from oxidative stress during maternal VitC deficiency.

Chapter 1 The biological function of Vitamin C

VitC is an important micronutrient involved in several molecular pathways and is considered to be an effective antioxidant required to protect from oxidative stress and its mediated macromolecular modifications [Lykkesfeldt et al., 1997; Tveden-Nyborg and Lykkesfeldt, 2013]. Recent cross sectional studies based on plasma VitC levels have shown that a substantial population is VitC deficient (not the disease, scurvy) even in the developed countries [Villalpando et al., 2003]. However, the molecular effects of such non-scorbutic VitC deficiency are not clear. Guinea pigs, like humans are dependent on dietary VitC and serve as a valuable animal model to investigate the effects of VitC deficiency [Lykkesfeldt et al., 2007; Yu and Schellhorn, 2013].

1.1.1 Vitamin C in antioxidant functions

VitC is well known for its ability to reduce other oxidizing compounds by donating electrons and thereby itself getting oxidized [Iyanagi et al., 1985; Linster and Van Schaftingen, 2007]. During this process VitC gets oxidized to ascorbyl radical (AR) following the loss of a single electron as shown in figure 1 [Padayatty et al., 2003]. Dehydroascorbic acid (DHA) is formed after the loss of a second electron which is unstable at physiological pH and temperature [Padayatty et al., 2003]. Presence of different reductase enzymes permits the reversible reaction of AR or DHA to ascorbate anion (ASC), which is the predominant form of VitC that exists at normal physiological conditions [Kobayashi et al., 1991; Linster and Van Schaftingen, 2007].

Biological functions of VitC can be specific and non-specific [Tveden-Nyborg and Lykkesfeldt, 2013]. VitC is involved in specific functions as a co-factor in the stabilization of collagen which is a major protein required for maintenance of connective tissue and wound healing [Kim and Peterkofsky, 1997], in stabilization of peptide hormones which is central for the processing of hormonal peptides in their active form [Murthy et al., 1987], in synthesis of carnitine that is needed for fatty acid transport into mitochondria for generating energy [Nelson et al., 1981], in norepinephrine synthesis which is an important catecholamine that regulates attention [Levine et al., 1985], in tyrosine metabolism for the synthesis of dihydroxyphenylalanine (DOPA) which is a precursor of catecholamine synthesis [Levine et al., 1941], in regulation of HIF-1alpha which is a transcription factor that activates diverse cellular pathways for survival under low oxygen

conditions [Goda et al., 2003] and in the reduction of tetrahydrobiopterin which is necessary for the function of nitric oxide synthase to maintain endothelial cell functions [Yan et al., 2012]. VitC in all these functions donates electrons and keeps iron or copper involved in these enzymes, which are either monooxygenases that incorporate a single oxygen molecule or a dioxygenase that incorporates two oxygen molecules, in their reduced forms in order to keep the enzyme in the active state [Gropper and Smith, 2012; Padayatty et al., 2003].

The non-specific role of VitC includes its function as a scavenger of reactive oxidizing agents like hydroxyl radicals, superoxide, peroxynitrite etc. to maintain cellular redox status [Buettner, 1993; Rose and Bode, 1993]. VitC has also been shown to recycle oxidized alpha tocopheroxy radical and this synergistic action maintains integrity of the cellular membrane in the event of redox imbalance [Chan, 1993]. Hence VitC is considered vital for several cellular functions and to protect the cell from oxidative stress and its mediated effects.

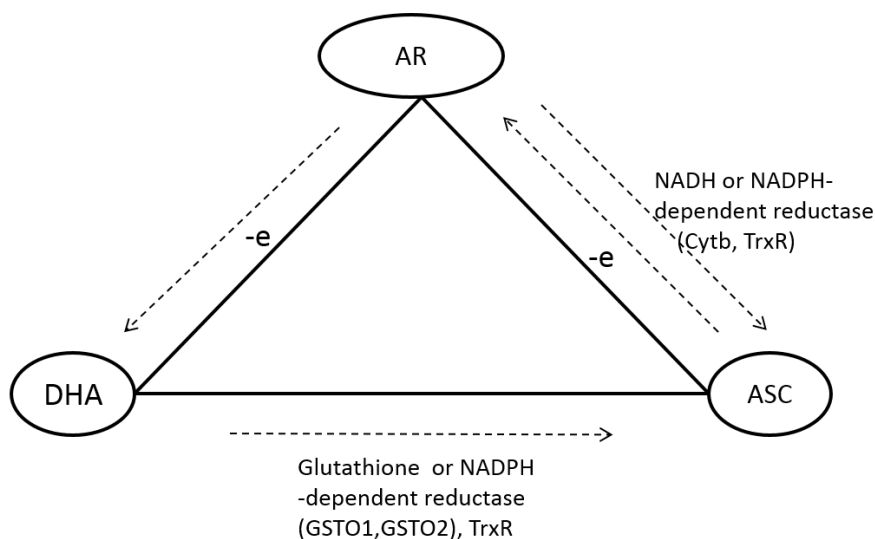


Figure 1 VitC regeneration (Modified from Rose et al., 1993)

Loss of an electron from ASC forms AR and loss of another electron from AR results in DHA. Glutathione dependent reductases (GSTO1, GSTO2) and NADPH dependent thioredoxin reductase (TrxR) reduces DHA to ASC. Similarly, NADH dependent cytochrome b reductase (cytb) or NADPH dependent thioredoxin reductases (TrxR) reduce AR to ASC.

ASC:ascorbate, AR:ascorbyl radical, DHA:dehydroascorbic acid

1.1.2 **Unchecked redox imbalance may modify macromolecules and cause apoptosis**

Biological systems are constantly exposed during aerobic metabolism to oxidants like free radicals that contain one or more unpaired electrons like superoxide anion ($\bullet\text{O}_2^-$), nitric oxide ($\text{NO}\bullet$), hydroxyl radical ($\bullet\text{OH}$) etc and oxidants that are not free radicals such as hydrogen peroxide (H_2O_2) [Finkel and Holbrook, 2000]. Some of the sources of biological oxidizing agents include mitochondria during aerobic respiration yielding $\bullet\text{O}_2^-$, $\bullet\text{OH}$ and H_2O_2 due to electron leakage to oxygen from intermediate coenzymeQ instead of at cytochrome oxidase at complex IV [Halliwell, 2006], neurotransmitter oxidation by monoamine oxidase, hypoxanthine oxidation during uric acid formation, fatty acid oxidation in the peroxisomes [Sheu et al., 2006], NADPH oxidases in macrophages during host defence and clearance of cellular debris [Lambeth, 2004] etc. However an increased production of cellular oxidants has been linked to pathophysiological conditions [Sies, 1997]. To counteract the effects of oxidants biological systems are protected by antioxidant mechanisms and most commonly include endogenously available enzymes like superoxide dismutase (SOD) which can be cytoplasmic, mitochondrial or extracellular specific that generates H_2O_2 by dismutation of $\bullet\text{O}_2^-$ [Zelko et al., 2002], catalase (CAT) and glutathione peroxidase (GPx) that decomposes H_2O_2 to water [Michiels et al., 1994]. Additionally, presence of non-enzymatic antioxidants such as VitC, vitamin E and glutathione play a vital role in quenching the increased oxidizing agents as shown in the figure 2. [Valko et al., 2007]. The synergistic action of VitC and vitamin E have been shown to result in an efficient inhibition of peroxidation [Niki et al., 1984; WEFERS and SIES, 1988].

Impaired ability to maintain the redox status (Figure 2) can have several consequences on macromolecular modification and may result in tissue damage. Macromolecular oxidation can be enhanced by interaction within free radicals, for example $\bullet\text{O}_2^-$ with nitric oxide ($\text{NO}\bullet$) forms peroxynitrite (ONOO^-) and modifies proteins by nitration [Hurst, 2002]. Alternatively, the products accumulating from membrane oxidation can be the precursors of DNA or protein oxidation [Hartley et al., 1999; Marnett, 2002]. One of the extensively studied measures of oxidative stress is lipid peroxidation that could result due to formation of hydroxyl radicals ($\bullet\text{OH}$) by Haber-Weiss reaction [Datta et al., 2000]. Lipid peroxidation is extensive in the presence of transition metals like ferrous

and cuprous ions [Halliwell and Chirico, 1993]. During the lipid peroxidation process, hydroxyl radical attack of the polyunsaturated fatty acids (PUFA) lets off a hydrogen atom from PUFA making it a lipid radical that can then react with oxygen to form lipid peroxy radical and can readily attack another PUFA [Betteridge, 2000]. This reaction of forming lipid radicals can continue resulting in the disruption of membrane integrity that may alter the functional state of the cell [Betteridge, 2000]. Lipid peroxidation gives rise to by-products like malondialdehyde (MDA) and hydroxynonenal (HNE) that are highly reactive [Halliwell and Chirico, 1993; Hoff and O'Neil, 1993] and can lead to further oxidation of macromolecules.

Oxidative stress has been associated with activation of caspases in the apoptotic signalling [Ikonomidou and Kaandl, 2011; Rees and Inder, 2005], however, the underlying mechanisms are not clear. Caspase-3 that can be activated by several mechanisms has been shown to be indispensable during development [Jänicke et al., 1998]. Apoptosis is a complex mechanism with different initiation signalling factors involved in mediating the energy dependent pathway of events and activation of caspase-3 is where the different pathways may converge [Elmore, 2007]. Caspase-3 activation results in DNA fragmentation and protein cross-linking during which several cytoskeletal and nuclear proteins are degraded and marked for phagocytosis [Schwartzman and CIDLOWSKI, 1993]. Both *in vitro* and *in vivo* studies have suggested the altered regulation of caspase-3 during different nutrient deficiencies [Bagnyukova et al., 2008; Cregan et al., 1999; Yamaguchi et al., 2001; Yen et al., 2002].

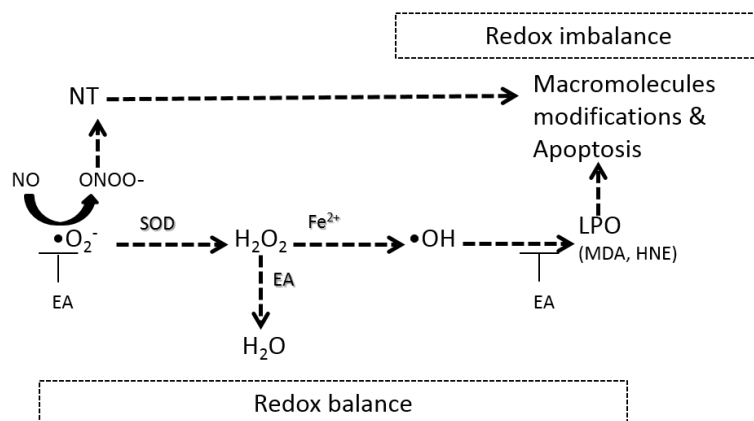


Figure 2 Cellular Redox homeostasis and oxidative stress

The increase in oxidizing agents can result in damage when the cellular antioxidants are unable to neutralize the increased free radical reactions as indicated by arrows leading to apoptosis. $\bullet\text{O}_2^-$

generated by cellular metabolism may either lead to formation of ONOO- that can modify macromolecules leading to apoptosis, or $\bullet\text{O}_2^-$ can result in increase of $\bullet\text{OH}$ when left unchecked by antioxidants leading to LPO. Conversely, endogenous antioxidants (EA) maintain homeostasis between oxidants and antioxidants by inhibiting further oxidation under normal physiological conditions.

NT:nitrotyrosine, ONOO-:peroxynitrite, NO:nitricoxide, $\bullet\text{O}_2^-$:superoxide, H_2O_2 :hydrogen peroxide, $\bullet\text{OH}$:hydroxyl radical, LPO:lipid peroxidation, MDA:malondialdehyde, HNE:hydroxynonenal

1.1.3 Vitamin C deficiency and its prevalence

Low levels of VitC for a prolonged period may lead to the clinical condition scurvy which is associated with fatigue, skin and dental abnormalities and impaired wound healing [Hirschmann and Raugi, 1999]. However scurvy is rarely seen even in the developing countries due to the access to VitC rich food sources [Hirschmann and Raugi, 1999] and since as little as 10 mg/day may prevent from the onset of this clinical condition [Jacob and Sotoudeh, 2002]. VitC deficiency has been classified based on VitC levels in the serum as severe ($<11\mu\text{M}$) and marginal ($11\text{-}23\mu\text{M}$) deficiency [Lykkesfeldt and Poulsen, 2010].

Results from analysis of serum VitC from a population study of Mexican infants and children have shown above 25% prevalence of both moderate and severe VitC deficiency [Villalpando et al., 2003]. A cross-sectional study from the Canadian population suggested that out of seven young

adults three exhibit sub-optimal VitC levels which was further associated with increased expression of markers related to chronic disease [Cahill et al., 2009]. A Brazilian cross-sectional study tried to establish relation between cord blood VitC and maternal characteristics and reported that cord blood VitC was associated to maternal VitC, VitC-rich food intake, *per capita* income and alcohol. VitC deficiency is especially prevalent in the people belonging to low socioeconomic groups which also reflects low VitC consumption during pregnancy to a greater extent in this subset of people [Oliveira et al., 2009]. These studies suggest a substantial prevalence of marginal VitC deficiency and a greater need to investigate the effects of low VitC.

1.1.4.1 Guinea pig as an animal model of Vitamin C deficiency

Guinea pigs like humans are exceptional species that lost the functional gene L-gulono-g-lactone oxidase (*Gulo*) during evolution [Nishikimi et al., 1992]. Therefore VitC cannot be synthesized in guinea pigs and must be supplied through diet as in the case of human beings [Nishikimi et al., 1992]. This helps to successfully manipulate VitC dosage in guinea pigs to study the effects of non-scorbutic VitC deficiency [Lykkesfeldt et al., 2007; Tveden-Nyborg et al., 2012]. Guinea pigs also express GLUT1 on the erythrocytes similar to humans while rodents capable of synthesizing VitC express GLUT4 suggesting that since guinea pig and humans evolutionarily lost the functional *Gulo* gene, guinea pigs may mimic human physiology more when investigating effects of VitC [Yu and Schellhorn, 2013]. These animals were shown to be non-scorbutic when treated with precursor substrate of gulo together with modified Gulo enzyme suggesting that in addition to non-functional Gulo gene the endogenous substrate levels necessary for the modified gene is very low. However, this method of injecting the modified enzyme has been shown to have drastic effects [Hadley and Sato, 1989] which could mislead the primary focus of investigation. Hence dietary intervention has been the suitable way of assessing the effects of vital role of VitC on different aspects of physiology in guinea pigs [Bell et al., 2001; Heinrich et al., 2008; Lykkesfeldt and Moos, 2005].

1.1.4.2 Guinea pig foetal brain maturity during late gestation

Guinea pigs have gestational length about 66 days with a rapid brain development termed as brain growth spurt occurring around gestational day 45 [Dobbing and Sands, 1970]. Therefore these animals serve as valuable models to assess the impact of nutritional deficiencies during brain

development in utero [Edwards, 1967; Suckow et al., 2011]. Some of the important events that take place during the early time points of the late gestation reflecting growth spurt in this species are maturation of neuronal nuclei, increase in dendrites, accumulation of Nissl substance and increase in enzymatic activity [Dobbing, 2008]. Guinea pigs are precocial and relatively mature at birth which is in agreement with the event of adult amount of DNA being achieved soon after birth. Since the increase of neurons to nearly adult numbers is reached before the multiplication of glial cells, the adult volume of neuronal nuclei in these species is achieved ~20 days before birth [Dobbing and Sands, 1970]. Hence deviation from normal development in the late gestation foetal brains may cause disruption in the neuronal development.

In addition, Na^+, K^+ -ATPase measured as an index of membrane function in developing brain of guinea pig fetuses at different gestational days resulted in a marked increase after GD45 and this increase was suggested to be coincidental to cholesterol content that peaks just before birth [Mishra and Delivoria-Papadopoulos, 1988b]. The authors suggested that increase of Na^+, K^+ -ATPase signifies a pre-natal functional maturation of membranes and that the enzyme activity is dependent on increase in cholesterol content, phospholipids and unsaturated fatty acids. Enzymatic activities of aerobic glycolytic metabolism were also shown to be increased ~15 days before birth and were similar to activity to the adult state which correlates well with pre-natal neurological competence [Booth et al., 1980]. Moreover, the enzymatic activities of antioxidants CAT, GPx, glutathione reductase and glucose 6-phosphate dehydrogenase that protect from redox imbalance were shown to be increased 15-20 days before birth [Mishra and Delivoria-Papadopoulos, 1988a] suggesting that guinea pig foetal brain is relatively mature in terms of combating oxidative stress during late gestation. These findings suggest that the developmental pattern in guinea pig differs from humans, by being relatively mature and precocial at birth, but is a better animal model than other common rodent models. However, to target the peak of neurogenesis period, VitC deficiency must be imposed during gestation in guinea pigs.

Chapter 2 Vitamin C transport

Both ASC and DHA are reported to be absorbed by enterocytes of the human intestine [Malo and Wilson, 2000]. Since Asc exists as an anion at physiological pH and as DHA is relatively less hydrophobic, neither of the VitC forms can directly pass through the cellular membranes. To a small extent VitC was suggested to cross the cell membrane through passive diffusion however, this mechanism of transport is of minor importance [Lindblad et al., 2013]. Hence the primary transport of VitC relies on the transporters in the cell membranes as discussed in the sections below. The efflux of VitC from intestinal cells to the blood is vital to reach target tissues but the mechanism is not clear [Wilson, 2005]. It was proposed that volume sensitive anion channels in the intestinal epithelial cells may contribute for ASC efflux into ECF from where ASC may enter blood plasma through discontinuities in the capillaries [Liang et al., 2001]. Findings from ASC transport in endothelial cells proposed trans-endothelial movement or para-cellular movement of ASC as a route to reach tissue interstitium [May and Qu, 2009; May et al., 2009]. ASC from blood circulation upon reaching the kidney is filtered by the glomerulus and most of the VitC is then reabsorbed in the proximal tubules which is crucial for maintaining VitC levels in the body [Wilson, 2005].

2.1.1 DHA transport

DHA transport occurs by facilitative diffusion through glucose transporters (GLUT) and the mechanism is Na⁺ independent [Liang et al., 2001]. Differential distribution of the facilitative glucose transporters GLUT1, GLUT3 and GLUT4 enable uptake of DHA across a concentration gradient into the cells followed by rapid reduction of DHA into ASC [Rumsey et al., 2000; Rumsey et al., 1997]. Most of the blood cells were reported to transport DHA by this mechanism with VitC in its reduced form trapped intracellularly [Agus et al., 1997]. Due to this reduction mechanism, low levels of DHA are maintained in the cells which generates a concentration gradient across the plasma membrane and favours DHA uptake into the cells [Wilson, 2005]. A study with human intestinal brush border vesicles from different parts of the intestines showed that DHA is absorbed through the entire small intestine. However, the affinity with which DHA is transported is much lower (Km in enterocytes ;0.8 mM) indicating that most of VitC absorption is in its reduced form [Malo and Wilson, 2000]. Since DHA concentrations are drastically lower than ASC (95% human plasma) under normal physiological conditions, GLUT mediated DHA transport is unlikely to be

sufficient to meet cellular needs [Liang et al., 2001]. Additionally, DHA uptake competes for GLUTs with glucose which is 1000-fold higher than the levels of DHA (Wilson 2002) suggesting that DHA transport under normal physiological conditions is a very minor contributor to total VitC homeostasis [Rumsey et al., 1997].

2.1.2 ASC transport

Two membrane isoforms were characterized for the transport of ASC, sodium dependent vitamin C transporter1 (SVCT1) and SVCT2 [Tsukaguchi et al., 1999]. The SVCTs transport L-ascorbate with a Na^+ : Asc stoichiometry ratio of 2:1, against a concentration gradient by active transport and achieve high intracellular levels [Godoy et al., 2007; Harrison and May, 2009]. Energy for the transport is dependent on Na^+/K^+ -ATPase and *in-vitro* studies have shown that incubation of cells with an inhibitor of Na^+/K^+ -ATPase completely abolishes ASC transport [Savini et al., 2008]. Other ions tested in place of Na^+ have also been shown to abolish uptake of VitC and decreased the transporters' binding affinity at a lower pH [Liang et al., 2001]. The amino acids sequence homology between human SVCT1 and SVCT2 is nearly 65% with altogether 12 transmembrane domains but do not share a similar homology with other sodium co-transporters [Tsukaguchi et al., 1999]. However, the two transporters differ in the mechanism by which they transport VitC based on their distribution in cells and by varying in the affinity and capacity with which they transport VitC [Rivas et al., 2008]. Cellular studies on intestinal epithelial cells and kidney proximal tubule cells have reported polar localization of the transporters with the presence of SVCT1 on the apical side and SVCT2 on the basolateral side [Boyer et al., 2005; Nualart et al., 2013; Savini et al., 2008] suggesting that both transporters are required for effective VitC transport in these cells. Boyer *et al.*, suggested that SVCT1 on the luminal or apical side contributes to VitC absorption and SVCT2 on the basolateral side involves in uptake of VitC from the blood to the intestinal cells [Boyer et al., 2005] indicating non-redundant functions of the transporters.

2.1.3 Physiological roles of SVCT1

SVCT1 is a high capacity/low affinity transporter (K_m 65–252 μM) and transports more VitC than needed to maintain homeostasis in the body [Corti et al., 2010; Lindblad et al., 2013; Wilson, 2005]. SVCT1 is predominant in kidney, liver and intestine and plays a vital role in reabsorption

and regulation of vitamin C levels in plasma. This was supported by a study involved with SVCT1 encoding gene knockout, *Slc23a1*^{-/-}, mice which were shown to have increased urinary loss of VitC due to the failure of kidneys to reabsorb VitC, resulting in significantly lower VitC levels in the plasma [Corpe et al., 2010]. In the same study VitC levels in the brain and adrenal was not effected suggesting retention of VitC by tissues predominant with SVCT2 distribution while other organs tested showed decrease in tissue VitC levels in the *Slc23a1*^{-/-} mice. SVCT1 expression in the apical membrane of proximal tubules of the kidney was reported to increase progressively in the developing postnatal mice suggesting that VitC reabsorption after birth is enhanced by an increased SVCT1 expression in the proximal tubules [Nualart et al., 2013]. Liang *et al* proposed that protein kinases effect the intracellular transport of VitC by increased removal of the transporter from the membrane or decreased membrane insertion of the transporter in an *in vitro* study [Liang et al., 2002] indicating that cellular metabolism may influence the function of SVCT1.

2.1.4 Physiological roles of SVCT2

SVCT2 is a low capacity/high affinity transporter (Km 8–69 μM) that enables efficient concentration of VitC by widespread uptake [Rivas et al., 2008; Savini et al., 2008]. SVCT2 has been detected in most tissues [Liang et al., 2001] and is vital for maternal transport of VitC to the foetus and VitC accumulation in the CNS [Sotiriou et al., 2002]. In-vitro studies on human trophoblasts and the first trimester chorionic villi were reported to express SVCT2 which is important for the transfer of maternal VitC to the foetus [Biondi et al., 2007]. SVCT2 expression has been suggested to protect from oxidative stress due to its vital role in the uptake of VitC in metabolically active tissues and specialized cells [Savini et al., 2008]. SVCT2 protein has several cysteines that may be susceptible to protein oxidation when there is an increase in the cellular oxidants [May, 2012]. An *in-vitro* study reported oxidative modification of sulfhydryl groups on SVCT2 following exposure with oxidizing agents [May and Qu, 2004]. Additionally, SVCT2 also has sites for the binding of activating protein-1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) transcription factors that are involved in transcription of redox sensitive genes [Savini et al., 2007b] suggesting that its regulation may be based on the redox status of the cellular environment. Two *in vivo* studies on ischemia have reported redox regulation of SVCT2 [Berger et al., 2003; Gess et al., 2011]. Both the studies have shown an increase of SVCT2 around the affected areas suggesting that elevated stress could up-regulate SVCT2 in the

brain as a compensatory mechanism. SVCT2 protein expression in the rat brain showed high levels of the transporter in cornu ammonis (CA) and dentate regions of the hippocampus as well as cerebral cortex which is in accordance with the analysed VitC levels, suggesting that the expression of this transporter may be prominent in the regions where antioxidants levels are present in high levels [Mun et al., 2006]. Deletion of gene encoding SVCT2 has been shown to result in undetectable levels of VitC in the brain and intra-cerebral haemorrhage leading to perinatal death [Sotiriou et al., 2002]. These findings show that SVCT2 is indispensable for VitC transport.

2.1.5 Transport of VitC in the brain

Since SVCT1 is not expressed in the CNS, SVCT2 is the critical transporter involved in VitC uptake in the brain [Berger and Hediger, 2000; Lee et al., 2003]. Tight junctions between endothelial cells of the blood brain barrier and the lack of SVCT2 in brain capillary endothelial cells makes VitC entry primarily thorough the choroid plexus epithelial cells where high levels of SVCT2 have been reported [Harrison and May, 2009]. VitC from the plasma is transported into the CNS by generating concentration gradients in two steps as shown in figure 3. The first concentration gradient is generated upon active transport of VitC by SVCT2 across choroid plexus epithelial cells [Rice, 2000; Spector and Lorenzo, 1973]. From the choroid plexus, ASC is transported to CSF where the levels of VitC are reached around the same as in ECF by diffusion. Transport of ASC from ECF into neurons is also mainly by SVCT2 that generates another sharp concentration gradient in the neurons by accumulating several fold higher intracellular VitC levels [Grünewald, 1993; Sotiriou et al., 2002].

Conversely glial cells have been reported to lack either of the SVCT transporters [Berger and Hediger, 2000]. However, DHA generated in the neurons leaves through GLUTs to the ECF and enters the glial cells which comprise GLUTs in their membranes [Harrison and May, 2009]. DHA is rapidly reduced to ASC in the glial cells and thus accumulated intracellular ASC can be released back into ECF by an unknown mechanism. DHA is toxic to the neurons in the absence of its clearance by the glial cells [Wilson, 2005]. DHA can also be transported directly through the GLUTs present in the blood brain barrier but due to its very low levels detected in the blood this mechanism of transport may be of little significance [Liang et al., 2001].

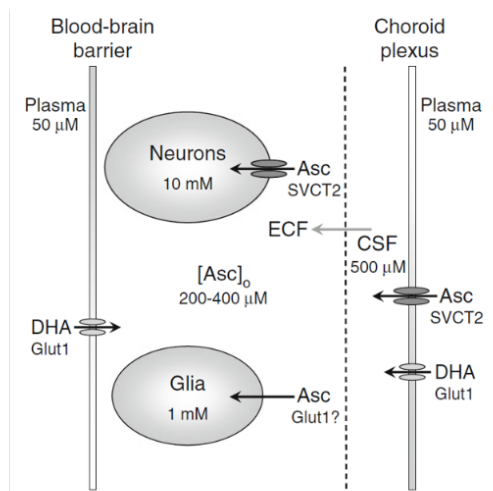


Figure 3 VitC transport in brain (Rice M E et al., 2009)

VitC in brain primarily enters in its reduced form ASC by active transport by SVCT2 at choroid plexus. First concentration gradient of ASC is created when ASC reaches CSF from plasma, accumulating 10 fold ASC than plasma. ASC from CSF diffuses to ECF. From ECF, ASC enters neurons through SVCT2 generating the second concentration gradient of ASC by accumulating millimolar VitC levels in the neurons. DHA can enter the brain through Glut1 on the blood brain barrier and similarly oxidized VitC in the ECF can make its way into neurons and glia through Glut1.

ASC:ascorbate, DHA:dehydroascorbic acid, Glut1:glucose transporter, ECF:extracellular fluid, CSF:Cerebrospinal fluid, SVCT2:sodium dependent VitC transporter2

2.1.6 Modulation of VitC transport

Reports from *in vitro* studies suggested that intracellular signalling may effect VitC transport. In human hepatic cell line SVCT1 was shown to be decreased by protein kinase C signalling and phosphorylation events resulting in down regulation of VitC uptake [Reidling et al., 2008]. Similarly increase in *Svct2* mRNA by glucocorticoids resulted in an increased VitC uptake [Fujita et al., 2001]. VitC uptake in intestinal cells derived from the hypervitaminosis guinea pigs was shown to be significantly reduced compared to the control group suggesting that VitC transport is effected by intracellular VitC levels [Karasov et al., 1991]. Depletion or repletion of VitC in the *in-vitro* studies showed modulation of VitC transport, with depletion of VitC resulting in an increased expression of the SVCT2 in the platelets and conversely addition of VitC levels resulting in a decreased SVCT1 expression in the intestinal cell line [MacDonald et al., 2002; Savini et al., 2007a]. Among the *in-vivo* studies, depletion of VitC in the *smp30/gnl^{-/-}* mice that cannot

synthesize VitC resulted in an increased mRNA of both SVCTs in the liver. The results reflected increased VitC uptake in the hepatocytes of these mice [Amano et al., 2010]. These findings suggest that intracellular VitC levels may regulate the transporters *in vivo* too. We have also shown SVCT1 up-regulation in the VitC deficient liver and an up-regulation of *Svct2* in the VitC depleted liver of the guinea pigs (Manuscript I- Figure 2). Based on this finding we suggested that SVCT1 may increase during chronic deficiency and SVCT2 may increase during severe deficiency of VitC. In contrast, our results from non-scorbutic VitC deficiency or depletion or repletion of VitC in guinea pigs reflected VitC levels in the brain (Manuscript I- Table 2 ; Manuscript II- Table 2) but did not show significant changes in the SVCT2 expression (Manuscript I – data not shown; Manuscript II- Figure1 & 3). Our findings suggest that low VitC levels in the brain may not regulate the overall SVCT2 expression in the brain and is in accordance with SVCT2 expression reported by others in VitC deficient *gulo*^{-/-} and *smp30/gnl*^{-/-} mice brain [Amano et al., 2010; Meredith et al., 2011].

Chapter 3 Roles of Vitamin C in the brain

3.1.1 VitC levels are differential in the brain

VitC in the brain has been shown to be several-fold higher than plasma indicating that high levels may be essential for proper functioning of the CNS [Harrison and May, 2009]. An earlier study in the guinea pig brains has reported nearly similar levels of VitC in the forebrain, midbrain and the hindbrain regions [Chinoy, 1972] and later it was suggested by Mefford *et al* that heterogeneous VitC levels in the brain are possibly due to the differences in neuronal innervations [Mefford et al., 1981]. In agreement high VitC levels were reported in the developing brain cortex of the new born rat during which time point neuronal cells are considerably higher compared to glial cells, suggesting preferential VitC localization in the brain to be a characteristic of neurons [Rice and Russo-Menna, 1997]. Hippocampus, the brain region associated with memory and spatial navigation was reported to have high VitC levels in the human, rat and the mouse brain [Harrison et al., 2010b; Mefford et al., 1981; Milby et al., 1982]. Along with the hippocampus, high VitC levels were reported in the cerebellum, olfactory bulbs and the frontal cortex and conversely low levels of VitC were reported from pons and spinal cord [Harrison et al., 2010b]. We have also shown differential VitC levels in the cerebellum and brain cortex of the guinea pigs (Manuscript I- Table 2). High levels of VitC in the cortex than cerebellum in our finding may be due to the differences in neuronal density in the analysed regions. These findings suggest that the role of VitC in the brain may be complex in-part due to differential levels within the brain.

3.1.2 Preferential retention of VitC in the brain

A consistent finding from VitC depleted guinea pigs and mice is that brain retains approximately 25% of VitC while the other organs are severely depleted underlining its preferential retention in the brain [Harrison et al., 2010b; Harrison and May, 2009; Hughes et al., 1971]. In accordance, our results show that non-scorbutic VitC deficient brain retains at least 30% of control VitC levels while kidney and liver showed 15-20% of control VitC levels (Manuscript I- Table 2). Moreover, VitC depletion resulted in drastically low retention of VitC in the kidney and liver with less than 2% of control levels while the brain retained 30% of control levels (Manuscript I- Table 2). Most importantly the depleted group had begun to lose body weight which is one of the early signs of

scurvy (Manuscript I- Figure 1) and yet the brain retained relatively high VitC levels. We showed similar VitC retention in foetal guinea pig brains at two time points of late gestation, pre-term/GD 45 and near-term/GD 56), due to low maternal VitC intake. (Manuscript III- Table 2). Also, the developmental decline of brain VitC at near-term as seen in the control group was not detected in the near-term deficient group signifying the requirement of maintaining optimum VitC levels during development. Hence, despite the preferential retention of VitC in the brain, non-scorbutic VitC deficiency or low maternal VitC intake may not be sufficient to tolerate the free radical mediated reactions that are suggested to be high in highly metabolic tissues like brain, and may lead to oxidative stress.

3.1.3.1 Low VitC intake imposes oxidative stress

We have previously shown that VitC deficiency in weanling guinea pigs significantly increased brain MDA levels compared to VitC sufficient controls [Lykkesfeldt et al., 2007]. Similar results were reported by others in VitC deficient *Gulo*^{-/-} mice, with elevated neuroprostane levels in both cortex and cerebellum compared to *Gulo*^{+/+} mice [Harrison et al., 2008]. The same authors demonstrated higher lipid peroxidation and a marked decrease of type IV collagen colocalization with laminin in *SVCT2*^{-/-} foetal cortex. However, immunohistochemistry revealed strong staining for type IV collagen in the endodermal membrane [Harrison et al., 2010a] and the authors suggested that unhydroxylated type IV pro collagen is retained due to defect in collagen secretion in the *SVCT2*^{-/-} brains that may have led to capillary haemorrhage. Additionally isoketal staining, another lipid peroxidation marker, and apoptotic cells were also reported to be high in the *SVCT2*^{-/-} brain [Harrison et al., 2010a].

Our results also showed an increase in lipid peroxidation marker, MDA, due to pre/post natal VitC deficiency (Manuscript II- Table 2) and in the late gestational foetal brains due to low intake of maternal VitC (Manuscript III- Figure 1B). In contrast, our results did not show significant changes in other analysed protein oxidation markers (Manuscript III- Figure 1A, 1C) or apoptotic marker caspase-3 (Manuscript III- Figure 2), between VitC control and deficient groups suggesting differential regulation of oxidative stress markers during development. In addition, SOD activity was increased in the VitC deficient foetal brain at both time points suggesting increase in the superoxide anion. Increased SOD activity together with elevated MDA levels suggests that

oxidative stress caused by non-scorbutic VitC deficiency may not be alleviated by compensatory mechanisms. Therefore, high levels of brain VitC are essential to protect from oxidative stress. Main findings from studies by others on the role of VitC, in protecting against oxidative stress induced by hypoxia, alcohol and neurotoxic compounds are discussed in the following sections to support the protective role of VitC. Based on our results and findings by others the effect of low VitC levels is graphically represented in figure 4.

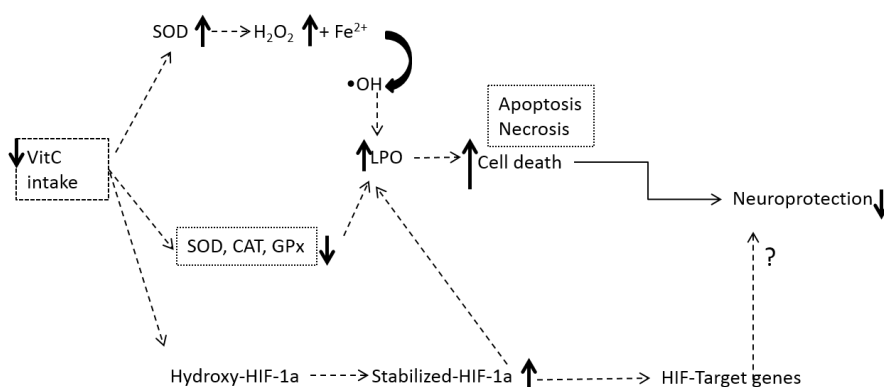


Figure 4 Effect of low VitC on oxidative stress

VitC deficiency has been shown to decrease enzymatic antioxidants SOD, CAT, GPx causing LPO. Alternatively increased SOD can result in increased H_2O_2 levels which in the presence of reduced iron (Fe^{2+}) can form hydroxyl radicals ($\bullet OH$) to initiate LPO. Low VitC may increase HIF-dependent genes together with increased LPO resulting in initiation of cell death and decreased neuroprotection. However, role of HIF-target genes during VitC deficiency is not known as shown by '?'. Both apoptosis and necrosis have been suggested to be the effected mechanisms by low VitC induced oxidative stress. SOD:Superoxide dismutase, CAT:catalase, GPx:Glutathione peroxidase, LPO:lipid peroxidation, H_2O_2 :hydrogen peroxide, HIF-1 α :Hypoxia inducible factor1-alpha,

3.1.3.2 Effect of VitC on hypoxia mediated oxidative stress

Role of VitC as one of the major antioxidants in the brain is supported by a study in pond turtles that have higher levels of VitC than rats and have a higher tolerance to hypoxia and the reoxygenation suggesting that high VitC levels compensate to prevent oxidative damage [Rice et al., 2002]. Lipid peroxidation markers in these species showed to be minimal during hypoxia or

reoxygenation compared to damage reported due to hypoxia or recovery in mammals [Willmore and Storey, 1997]. VitC was shown to sharply rise in the voltammetric measurements from rat hippocampus when hypoxia was induced suggesting VitC as vital free radical scavenger [Cammack et al., 1992]. In a primate animal model the effect of pre-treatment of VitC on middle cerebral artery occlusion (MCAO) was shown to decrease the infarct sizes significantly smaller, suggesting that VitC has a potential role in reducing infarction [Ranjan et al., 1993]. Similarly, intraperitoneal injection of VitC (500mg/Kg) in gerbils before and after bilateral carotid artery occlusion (BCAO) reduced neuronal damage modestly in hippocampus and significantly in striatum as assessed by histology [Stamford et al., 1999]. Another in-vivo study by Miura et al demonstrated neuroprotective effect of VitC following cerebral ischemia in rat pups by showing fewer fodrin break down products that signifies suppression of calpain activation resulting in lesser necrosis and damage [Miura et al., 2006]. In a later study in rat pups intraperitoneal injection of VitC (750 mg/Kg) before and after hypoxia significantly decreased macroscopic brain injury, decreased both necrotic and apoptotic cells in the different regions of the brain including hippocampus, suggesting that neuroprotective role of VitC partly includes inhibition of caspase-3 and calpain activation [Miura et al., 2009]. As mitochondria are the major sources of free radical production, transient ischemia was proposed to result in disruption of mitochondrial function and VitC treatment in dams before intrauterine ischemia was reported improve mitochondrial respiratory function in the foetal brain homogenates [Nakai et al., 2002]. Under normal conditions hypoxia inducible factor (HIF)-1 α is hydroxylated by a prolyl-hydroxylase with VitC as a co-factor resulting in hydroxylated HIF-1 α which is targeted to proteosomal degradation to control the regulation of HIF targeted pathways [Wenger et al., 2005]. Results from an in-vitro study showed that low VitC negatively affects prolyl hydroxylases by which increased stabilization of HIF- α can occur with the result of transcriptional activation of several target genes involved in glycolysis, cell survival etc. [Nytke et al., 2007]. However, a similar mechanism of VitC *in vivo* is not known.

3.1.3.3 Effect of VitC on ethanol induced oxidative stress

Voltammetry experiments in the rat brain demonstrated a dose dependent rise in VitC levels in the ECF in nucleus accumbens and striatum by intraperitoneal injections of ethanol [Svensson et al., 1992] suggesting that brain VitC levels can be effected by ethanol. Increased lipid peroxidation was reported in the rat cerebellum that was accompanied by significant decrease in VitC levels

following an intraperitoneal administration of ethanol [Rouach et al., 1987] suggesting that VitC is consumed in the process of free radical scavenging. Consumption of alcohol during pregnancy has been shown to result in foetal alcohol syndrome that affects the brain of the foetus by marked reduction in size (microencephaly) and retardation of growth and studies supporting this hypothesis have shown that excessive alcohol induces neurodegeneration accompanied by formation of free radicals [Haorah et al., 2005]. The protective effect of VitC was demonstrated by a reported reduction in ethanol induced microencephaly and growth retardation in the embryos treated with VitC before or concurrently with ethanol [Peng et al., 2005]. The forebrain size in this study was restored to near-control values when pre-treated with VitC and histological assessment reported nearly 50 % of neurons to be abnormally large when untreated with VitC. VitC pre-treatment suppressed H₂O₂, MDA, transcription levels of NF- κ B and restored neural markers as a protective mechanism [Peng et al., 2005]. In another study, SOD and catalase activities were elevated as a protective mechanism in the VitC treated guinea pig brain, suggesting modulation of endogenous antioxidants by VitC to reduce ethanol effects [Suresh et al., 1999].

3.1.3.4 Effect of VitC on oxidative stress induced by toxic compounds

Toxic effects of 1-methyl-4-phenyl pyridinium ion which was mainly promoted by depletion of dopamine and degeneration of striatal neurons by promoting lipid peroxidation was shown to be significantly reduced by intraperitoneal administration of VitC before the administration of the toxin [Wagner et al., 1986]. Similarly neurotoxicity caused by 3,4-ethylenedioxymethamphetamine (MDMA) has been shown to increase free radical mediated decrease in tissue 5-HT (5-hydroxytryptamine or serotonin) which was shown to be prevented by concomitant injections with VitC in the rat striatum [Shankaran et al., 2001]. A recent study in rats have shown that VitC administration after MDMA treatment downregulates GFAP expression and limited the reduction of ATP and 5-HT [Li et al., 2006]. May *et al* showed that one of the ways that VitC protects from neurotoxicity at least in part is by sparing alpha tocopherol and decreasing lipid peroxidation in the neuronal cells [Li et al., 2003]. The effects of VitC against toxic compounds in the brain was also shown by restoration of acetyl cholinesterase activity which is vital in the regulation of cholinergic synapses [Ambali et al., 2010], in preserving normal morphology of the neurons [El-Hossary et al., 2009], and by increasing endogenous antioxidants like SOD, CAT, GPx and glutathione reductase [Muthuvel et al., 2006]. Additionally, neuroprotective effect of VitC against toxic compounds was

associated with increased expression of anti-apoptotic genes that may enhance cell viability [Venkataraman et al., 2007].

3.1.4 Markers in neuronal cells development and maturation

Cellular development in CNS involves progression of cellular events like proliferation, differentiation, migration and maturation in order to determine the fate of the neuron and its location to interact with other neuronal circuits [Meller and Tetzlaff, 1975]. Differentiation of the CNS precursors can give rise to neurons and glial cells [Temple, 2001]. Differentiated neuronal lineage cells express doublecortin (DCX) and polysialylated embryonic form of the neural cell adhesion molecule (PSA-NCAM) and are referred to as immature neurons [und Halbach, 2007]. Immature neurons move from the midst of proliferative and differentiated cells and this stage aids in neuronal migration and DCX is the most popular marker of interest at this stage [Gleeson et al., 1999]. A study from neuronal cultures of dentate gyrus origin has shown that DCX supports the dendrites in elongation and reduced expression of DCX reduces dendritic motile structures called filopodia [Cohen et al., 2007]. Migrating neurons mature by extension of dendritic and axonal processes and this filopodia enhanced movement enables them to reach destined locations to make synaptic connections with other neuronal processes [Rakic, 1990]. The molecular mechanism of neuronal migration involves organization of cytoskeletal proteins with microtubule associated proteins (MAPs) like DCX leading to polymerization and stabilization of microtubules and actin filaments for proper orientation of the dendritic processes and filopodia [Feng and Walsh, 2001]. During this process a complex range of events ensure phosphorylation of MAPs and regulation of kinases like cyclin dependent kinase 5 (Cdk5) by regulator proteins such as P35 [Chae et al., 1997; Tanaka et al., 2004] and (brain1) Brn1 [Sugitani et al., 2002] to prepare the new neurons to correct locations as dictated by extracellular signalling cues [Ayala et al., 2007]. Finally, expression of neuron specific nuclear protein (NeuN) by the post mitotic neurons signifies maturation of neurons [Sarnat et al., 1998]. VitC effects on cellular events has been summarized in figure 5 based on results from the studies discussed in the following sections.

3.1.4.1 Effect of VitC on proliferation

Analysis of the stage specific markers by incorporation of synthetic nucleotide analogs during cell division like bromodeoxyuridine (BrdU) and analysis of stage specific markers has been popular

methods to investigate cellular development in the CNS [Ming and Song, 2005]. The CNS precursor cells divide and express their phenotype specific markers like nestin and glial fibrillary acidic protein (GFAP) together with endogenous cell division markers [Christie and Cameron, 2006]. Administration of consecutive BrdU injections to neonatal guinea pigs from P5-P9 that were assigned to VitC sufficient, deficient, repletion and depletion groups and assessment of proliferation and survival of the BrdU positive cells in hippocampus showed no significant differences [Tveden-Nyborg et al., 2012]. These results suggest that prenatal VitC deficiency does not affect postnatal newborn cells in the hippocampus. The results of Tveden-Nyborg *et al* were in line with an *in vitro* study which investigated effects of VitC in CNS precursor cells isolated from embryonic cortex of rat brain that showed no differences in proliferation in the cells treated with or without VitC [Lee et al., 2003]. In another study, significant inhibitory effect of VitC was seen on proliferation of astrocytes that were cultured from new born rat [Cheng et al., 1988] while the opposite effect was observed for VitC in rat embryonic mesencephalic cultures which showed a marked increase of GFAP [Kalir and Mytilineou, 1991]. These contrasting results were suggested to have been due to difference in the source of the tissue, being embryonic or neonatal brain tissue, employed in the studies [Kalir and Mytilineou, 1991]. Therefore due to limited reports, effect of VitC on proliferation in CNS remains inconclusive.

3.1.4.2 Effect of VitC on differentiation and maturation

Our earlier VitC dietary intervention study has shown a negative effect of VitC on migration of new born cells into the granular layer of hippocampal dentate gyrus [Tveden-Nyborg et al., 2012]. Analysis of the percentage of BRDU positive cells in sub-regions of the dentate gyrus in the prenatally VitC deficient guinea pig brains, showed significantly lower number of cells in the granule layer and significantly higher in the subgranular layer, compared to controls with sufficient VitC. Our preliminary results from mRNA expression analysis of neuronal migration markers *brn1*, *p35*, *cdk5*, *dex* from VitC control, deficient and repleted hippocampus of guinea pigs did not show significant differences (data not published) indicating that the observed delayed migration is complex to be detected by mRNA analysis.

VitC has been shown to enhance neuronal differentiation from mouse embryonic stem cells and the differentiated neurons in presence of VitC in the culture media had increased neurons that were

positive for Tyrosine hydroxylase (TH), the enzyme responsible for the formation of the precursor of dopamine [Lee et al., 2000]. Consistently with the increase of TH positive neurons, dopamine levels were also increased in the media that had VitC and further analysis determined these neurons as functional [Lee et al., 2000]. Another study performed in embryonic rat mesencephalic cell cultures also showed a similar finding that addition of VitC in the medium significantly increases TH positive neurons compared to the cultures without VitC. This study additionally showed that VitC also increases serotonergic neurons and the authors suggest that VitC may activate cell differentiation that effects dopaminergic neuronal yield [Yan et al., 2001]. Yu *et al* showed that VitC treated CNS precursor cells derived from rat embryonic mesencephalon had increased expression of neuronal (MAP2a and MAP2b) and astrocytes (GFAP) markers indicating that VitC stimulates formation of both neurons and astrocytes [Yu et al., 2004]. In this study, the yield of dopaminergic neurons was very low when untreated with VitC. In contrast, when VitC was added to the medium during proliferative or differentiation phases of the cells, increase in TH positive neurons was markedly significant. This study confirmed up- regulation of tyrosine hydroxylase by both gene and protein expression, in response to VitC treatment in the cells, suggesting transcriptional and translational effect of VitC on dopaminergic neuronal differentiation [Yu et al., 2004]. However, the up-regulated molecular markers identified by cDNA micro array analysis were different from the markers that were known to play an important role in the midbrain and the authors suggested that VitC may promote dopaminergic neuronal differentiation by uncharacterized pathways [Yu et al., 2004]. Similarly, VitC treated CNS precursor cells from rat embryonic cortex showed enhanced effect of VitC on differentiation to neurons and astrocytes as measured by neuronal Tuj1 and astrocytic GFAP positive cells and morphology of the VitC treated cells were more mature with longer extended processes [Lee et al., 2003]. Additionally, Lee *et al* gave insights on the molecular mechanisms involved in the process of VitC enhanced differentiation by investigating mRNA expression of some of the known genes vital to differentiation into neurons and astrocytes. Their results showed that presence of VitC in the cultures increased four out of twenty genes investigated. Among them are NeuroD and Notch, markers involved specifically in neuronal and astrocytes differentiation, respectively. Interestingly VitC treatment promoted numerous extended processes on the cells which were in coherence with increased miniature post synaptic currents (mEPSCs) indicating that VitC has a positive effect on synaptic maturation of neurons [Lee et al., 2003]. Immunohistochemistry with anti-SVCT2 on hippocampal neurons from embryonic mice were shown to have intense SVCT2 in axons and faint signal in the soma [Qiu et

al., 2007]. Since, SVCT2 is necessary for the cells to maintain intracellular VitC, additionally experiments on cultured hippocampal neurons that were derived from SVCT2 knock out embryonic mice and embryonic wild type mice, were compared for mEPSCs, immunostaining of MAP2 which is a marker of dendrites and GluR1 which is an important glutamate receptor and a morphological correlate of mEPSCs. It was observed that hippocampal SVCT2 wild type neurons had increased amplitudes with smaller intervals, complex dendrites which were more in number and longer in length and consisted of significantly more GluR1 clusters suggesting that VitC is vital for complex neuronal morphology and glutamatergic function of the neurons.

In addition to the formation of dendritic extensions, maturation of neurons involves a vital mechanism of myelination which influences the speed with which the neuronal impulses are transmitted [Stokes, 2004]. Stevens *et al* demonstrated that neuronal impulse activity that has low-frequency will have significantly reduced myelination in the neurons derived from dorsal root ganglia of the embryonic mice [Stevens et al., 1998] suggesting that the effect of VitC on neuronal maturation also includes myelination of neurons. Since the few existing data regarding effect of VitC on myelination are from experiments on Schwann cells, the main findings from these are discussed here. Addition of VitC in the medium has been shown to be a requirement for differentiation of Schwann cells, and VitC promoted the cells to aggregate together on the surface of axons and most importantly deposition of laminin, collagen type IV and heparin sulphate proteoglycan (HSPG) were observed only when VitC was included [Eldridge et al., 1987]. Carey and Todd *et al* have suggested that ensheathment and myelination of axons is dependent on extracellular matrix formation and demonstrated it in VitC treated Schwann cells and neurons derived from embryonic rat dorsal root ganglia (DRGN) [Carey and Todd, 1987]. Similarly, DRGN derived from adult rats that were co-cultured with Schwann cells in the absence of VitC showed no myelin segments [Plant et al., 2002]. This study also showed a marked difference in the morphology of the ensheathment of myelin in the VitC treated cells that revealed axons encircled by schwann cell cytoplasm [Plant et al., 2002]. In accordance with these findings, dietary intervention with VitC was shown to partially correct the pathology of neuropathy in mice by remyelination of the axon fibers [Passage et al., 2004]. Histological assessment of peripheral nerves of the mice that were treated with VitC showed a 50% higher myelination and their sheath thickness was significantly greater than the nerves from mice treated with placebo [Passage et al., 2004]. Data from all these studies support an enhancing effect of VitC on neuronal maturation.

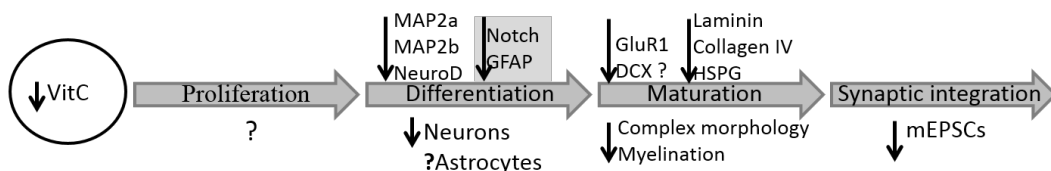


Figure 5 Effect of VitC on cellular events in CNS

VitC deficiency has been shown to effect at different stages of cellular development. Effect of low VitC on proliferation is inconclusive. Low VitC may effect differentiation of cells into neuronal lineage or may cause deficits in the collagen, myelination or migration of neurons that may result in impaired synaptic integration with other neurons.

Black solid arrows indicate decreased/down regulation. Assessed molecular markers are indicated above the specified event with neuronal markers unshaded and astrocyte markers in grey shaded box. GluR1: Glutamate receptor, DCX: Doublecortin, HSPG: heparin sulphate proteoglycan, mEPSCs: miniature post synaptic excitatory currents '?' : inconclusive.

3.1.5 Effect of VitC on glutamate re-uptake

VitC from neurons and astrocytes has been shown by in-vitro studies to efflux into the extracellular space. This release of VitC into the ECF aids in the clearance of glutamate from around the synaptic clefts and protects the neurons from excess glutamate which would otherwise be detrimental [Rice, 2000]. When neuroblastoma cells were incubated with increasing concentrations of glutamate, the transport of radiolabelled [^{14}C] VitC into the cells was shown to be inhibited and a higher efflux of the radiolabelled VitC in the medium was reported as a measure of both disappearance of VitC from the cells and by its appearance in the culture media [May et al., 2006]. Intrastriatal infusion with glutamate in rats were shown to result in increased release of VitC levels for short time [Pierce and Rebec, 1993]. Activation of N-methyl-D-aspartate receptor (NMDAR) by glutamate release is essential for excitatory synaptic transmission and synaptic plasticity but excessive stimulation of these receptors results in neurotoxicity [Paoletti and Neyton, 2007]. Re-uptake of glutamate mediated increase in extracellular ascorbate was shown to be independent of NMDAR for activation as NMDA antagonists did not have an effect on glutamate induced VitC release in striatum [Pierce and Rebec, 1993]. However, the role of glutamate re-uptake sites in the release of

VitC was supported by a study that showed rapid VitC efflux following glutamate injections in different brain regions and the efflux of VitC was completely obstructed when both glutamate and glutamate uptake inhibitors of neurons and glia were co-injected [Cammack et al., 1991]. Additionally in this study, co-injecting glial specific glutamate uptake inhibitor alone and glutamate demonstrated an incomplete obstruction of VitC release suggesting that neurons like glia have an important role in the reuptake of glutamate. Similarly it was demonstrated that VitC release from striatum is blocked when the rat brain was perfused with voltage gated calcium channel blocker or glutamate uptake blocker, supporting that the release of VitC is dependent on glutamate uptake and calcium ions [Miele et al., 1994]. In neuronal cultures derived from rat cortex, the cultures treated with VitC markedly reduced cell death following treatment with high levels of NMDA or glutamate [Majewska and Bell, 1990]. Majewska *et al* proposed a mechanism of VitC in preventing excessive activation of NMDA receptors by altering the density of glutamate recognition sites on these receptors in isolated neurons [Majewska et al., 1990]. Efflux of VitC has been suggested as a mechanism to protect from excitotoxic effect of glutamate during stressful conditions like trauma where efflux of high glutamate levels have been associated to the increase in free radicals [Hillered et al., 1990; May et al., 2006]. During ischemia-reperfusion in rats it was demonstrated that extracellular VitC increases briefly during ischemia and linearly increases after reperfusion while glutamate levels increased linearly from the beginning of ischemia after which they decreased rapidly [Yusa, 2001], suggesting a VitC-glutamate heteroexchange mechanism in which glutamate uptake into cells parallels extracellular release of VitC. Although the mechanism of VitC efflux is not known these studies help to understand the importance of intracellular VitC stores in regulation of extracellular glutamate. Figure 6 graphically represents VitC deficiency in leading to glutamate toxicity around neurons.

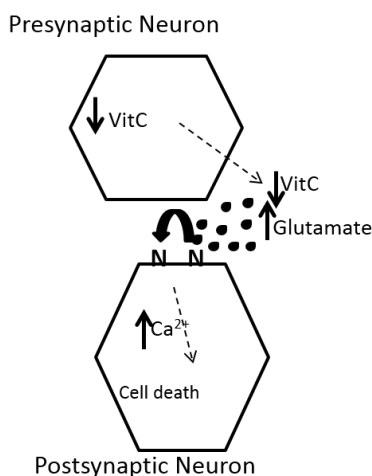


Figure 6 Effect of VitC deficiency on glutamate re-uptake
 Low intracellular VitC may be insufficient to efflux from the neurons due to which glutamate may accumulate (solid dots) in the extracellular space causing an impaired re-uptake of glutamate by presynaptic neurons. This could cause an excessive activation of N (N-methyl-D-aspartate receptor/NMDAR) resulting in increased calcium ions influx that can likely be detrimental to neurons.

3.1.6 Effect of VitC on catecholamines

VitC has been shown to efficiently participate in the metabolism of catecholamines in several *in vitro* studies. In human neuroblastoma cells VitC was shown to increase the levels of precursor of dopamine, dihydroxyphenylalanine (DOPA), dopamine and noradrenaline [Seitz et al., 1998]. Additionally, gene expression analysis of the neuroblastoma cells that were treated with VitC showed a significant increase in tyrosine hydroxylase suggesting that VitC increases catecholamine levels by up-regulation of tyrosine hydroxylase [Seitz et al., 1998]. The finding of Seitz et al was in coherence with another *in-vitro* study in human neuroblast cells that reported a translational increase in tyrosine hydroxylase with increase in intracellular VitC [May et al., 2012a]. The detected norepinephrine was very low in these cells when VitC was not added and a single addition of VitC resulted in time-dependent increase in norepinephrine levels suggesting dependence of norepinephrine levels on intracellular VitC. The authors ruled out pro-oxidant nature of VitC in this study as the observed increase in norepinephrine level did not differ in the cell cultures that were

additionally treated with antioxidants, SOD or CAT [May et al., 2012a]. VitC was also shown to enhance norepinephrine synthesis directly when dopamine was added to neuroblast cells [May et al., 2012b]. Among the in-vivo studies, Meredith *et al* investigated the effects of VitC on neurotransmitters in SVCT2 knock out embryonic mice and a transgenic mice model with increased SVCT2 [Meredith and May, 2013]. Their findings suggested that intracellular VitC levels determine some of the neurotransmitters *in vivo*, by showing lower levels of dopamine and norepinephrine together with tyrosine hydroxylase protein in SVCT2^{-/-} embryonic cortex compared to SVCT2^{+/+} and SVCT2^{+/-} genotypes. Lack of significant differences for serotonergic system in SVCT2^{-/-} was presumed as due to maintenance of tryptophan hydroxylase by adequate tetrahydrobiopterin reduction by other antioxidants like glutathione, even during VitC deficiency in the developing brain. Transgenic embryonic cortices with extra copies of SVCT2 showed significantly high dopamine, norepinephrine and serotonin compared to wild type however, they did not differ in tyrosine hydroxylase protein. In contrast, whole brains from SVCT2^{-/-} embryonic mice from another study did not show significant differences in dopamine and norepinephrine and the authors suggested that dopamine β-hydroxylase can function even during severe VitC deficiency in the developing brain and that it may not be the cause of lethality observed in SVCT2^{-/-} pups [Bornstein et al., 2003]. Similarly, a recent in-vivo study performed on Gulo^{-/-} mice showed that VitC deficiency in the cortex results in non-significant decrease of dopamine levels but dopamine metabolites, dihydroxyphenylacetic acid (DOPAC), 3methoxytyramine (3MT) and homovanillic acid (HVA), decrease significantly suggesting that VitC deficiency may alter dopamine catabolism [Ward et al., 2013]. A significant decrease in norepinephrine but not in dopamine levels has been reported in VitC deficient guinea pigs [Kaufmann et al., 1986]. Based on these findings as presented in figure 7, VitC deficiency may have variable effect on catecholamines depending on the age and type of animal model as well as the severity of VitC deficiency.

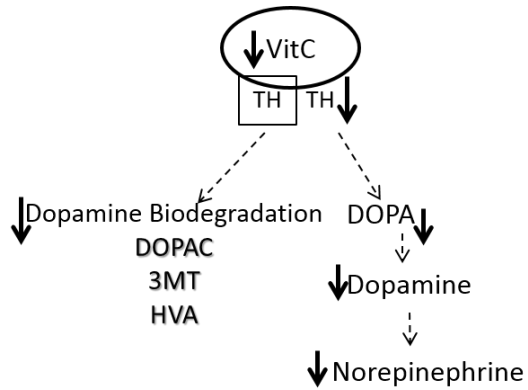
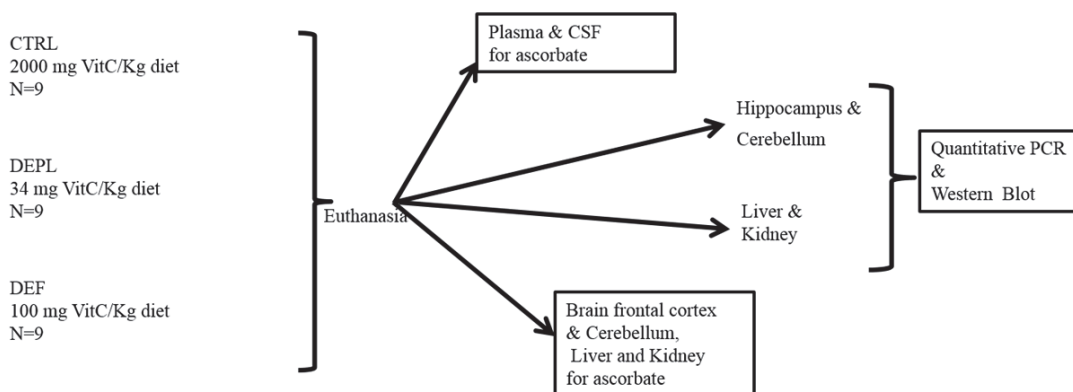


Figure 7 Effect of low VitC on catecholamines
 VitC deficiency can either decrease catecholamines TH dependently or alter dopamine biodegradation TH independently. Solid black arrows indicate regulation and dashed arrows indicate the pathway.
 TH: Tyrosine hydroxylase, DOPAC: dihydroxyphenylacetic acid, 3MT: 3methoxytyramine, HVA: homovanillic acid

Chapter 4 Graphical representation of studies

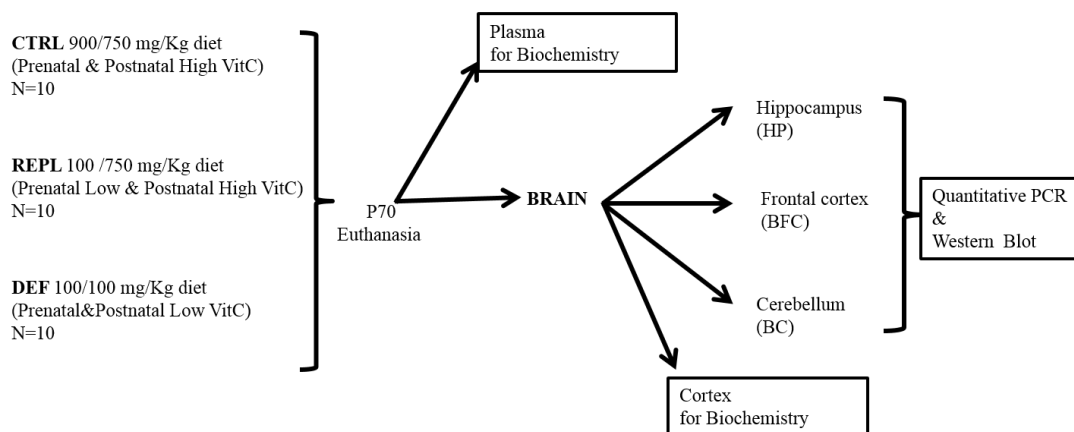
This section presents graphical representation of the studies that have formed the three manuscripts appended in this thesis. This is to provide a simplified picture of the number of animals in the study, dietary groups, different samples and the markers analysed. Our hypothesis in manuscript I was that during VitC deficiency high brain VitC relative to other organs is maintained in guinea pigs through induction of SVCT2. To test this hypothesis, we investigated regulation of SVCT2 expression following VitC deficiency as well as depletion, together with analysis of VitC transport from plasma to the CSF. Based on our earlier findings in which prenatal VitC deficiency was shown to result in an impaired brain development [Tveden-Nyborg et al., 2009; Tveden-Nyborg et al., 2012], we investigated the effect of prenatal VitC deficiency in both manuscript II and manuscript III. However, in manuscript II, the long term effect of prenatal VitC deficiency on oxidative stress was assessed by investigating in the postnatal guinea pig brain during deficiency and repletion. While in manuscript III, the short term effect of prenatal VitC deficiency on oxidative stress was assessed by investigating in the foetal brains during late gestational time period. Our hypothesis that led to manuscript II was that despite preferential retention of brain VitC, non-scorbutic VitC deficiency increases oxidative stress in guinea pigs. Our hypothesis that led to manuscript III was that the fetus is not protected from oxidative stress during maternal VitC deficiency.

Manuscript I Study Design



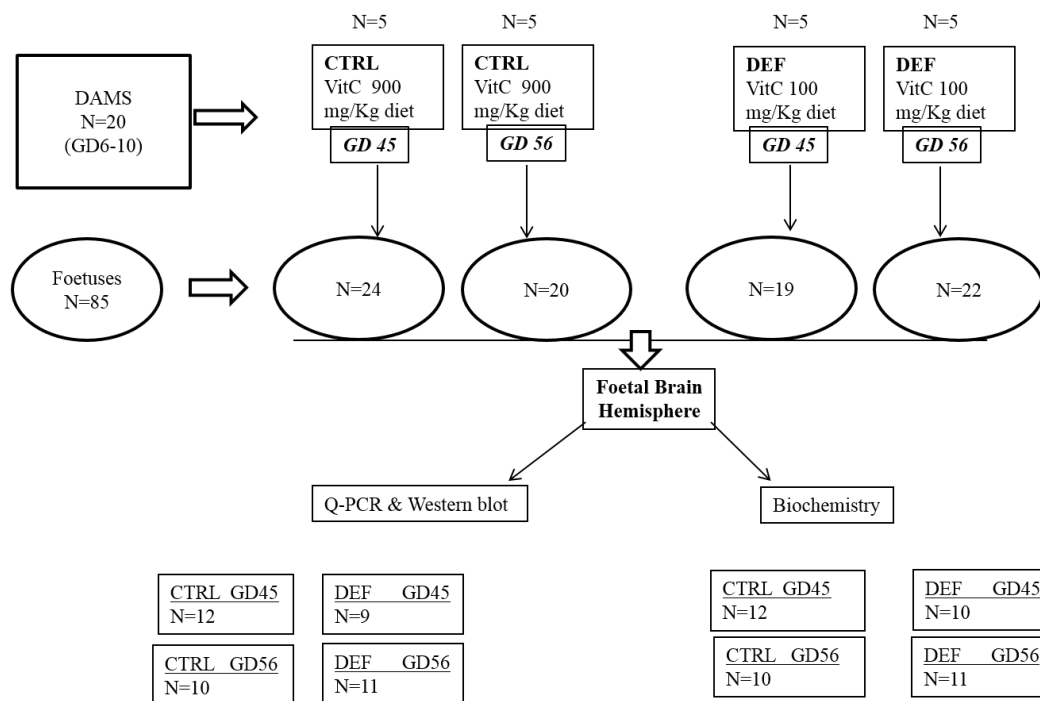
The study for manuscript I comprises twenty seven female (6-7 days old at the start of the study) Dunkin-Hartley guinea pigs that were randomized into three weight stratified groups (n=9/group). Control group (CTRL) received 2000 mg VitC/Kg diet, depletion group (DEPL) received 34 mg VitC/Kg diet and deficient group (DEF) received 100 mg VitC/Kg diet. At the end of the study animals were euthanized and VitC analysis was performed on plasma and cerebrospinal fluid (CSF), frontal cortex and cerebellum from brain, liver and kidney. mRNA and western blotting analysis of Svct1 and Svct2 was performed on Hippocampus, cerebellum, liver and kidney.

Manuscript II Study Design



The study which formed manuscript II comprises thirty female Dunkin-Hartley guinea pigs that were a subset of a large invivo study (Tveden-Nyborg *et al* 2012). The animals were divided to three dietary groups that differed only in the content of VitC (N=10/group). VitC content in the control feed was 900 mg/Kg diet pre-natally and 750 mg/Kg diet postnatally (pre-/postnatally sufficient, CTRL, 900/750 mg vitC/kg diet), VitC content in the deficient group was 100 mg/Kg diet both pre-natally and postnatally (pre-/postnatally deficient, DEF, 100/100 mg vitC/kg diet) and VitC content in the repleted group was 100 mg/Kg diet pre-natally and 750 mg/Kg diet postnatally (prenatally deficient/postnatally sufficient, REPL, 100/750 mg vitC/kg diet). At the end of the study around postnatal day 70, the animals were euthanized and hippocampus, frontal cortex and the cerebellum from randomized brain hemisphere (left or right) was used for mRNA and western blot analysis of Svct2. Plasma and brain cortex were used for biochemistry analysis which include ascorbate, dehydroascorbic acid, glutathione and malondialdehyde.

Manuscript III Study Design



The study that formed manuscript III comprised twenty pregnant guinea pigs between gestation day (GD) 6-10 and the dams were randomized according to GD and body weight into two dietary groups (N=10/group). Dietary groups were Control: CTRL (900 mg/kg diet) and Deficient: DEF (100 mg/kg diet). At euthanasia three dams, one from CTRL group and two from DEF group were found not to have conceived and were excluded from the study. Pups were taken out by caesarean at GD 45 (from 5 dams of CTRL GD 45 ; 4 dams of DEF GD 45) or GD 56 (from 4 dams of CTRL 56 ; 4 dams of DEF 56). Brains were removed out of the cranium and the left hemisphere from half of the total number of brains was used for analysis of ascorbate, malondialdehyde, alpha and gamma tocopherols and superoxide dismutase activity. The left hemisphere from the other half of the brains were used for analysis of mRNA and protein expression of caspase-3 and for western blotting to detect proteins modified by nitrotyrosine or hydroxynonenal.

Chapter 5

Discussion

5.1.1 Brain VitC is retained by unknown mechanism

Our results suggest that the overall regulation of SVCT2 may not be the mechanism by which VitC is retained in the brain. The hypothesis 1 is hence rejected. Our results are in agreement with other *in vivo* studies [Amano et al., 2010; Meredith et al., 2011] who have shown the same by mRNA and protein analysis in the mice brain. VitC enters the brain CSF primarily through the choroid plexus [Rice, 2000]. Based on our data on CSF VitC levels, (Manuscript I) 3.5 fold higher VitC than plasma was always seen irrespective of differences in the VitC diet. This finding suggests that there may not be differences in the transport rate of VitC in the brain even during deficiency. However, it remains unknown if regulatory mechanism of SVCT2 may be a possibility in choroid plexus itself or if active function of the transporter is increased, for instance by membrane insertion as shown for SVCT1 [Liang et al., 2002], in this region.

5.1.2 Retention of VitC or other compensatory mechanism in the brain does not protect from oxidative stress induced by vitamin C deficiency

VitC is suggested to be essential for survival as knock out of the gene encoding Svct2 results in death soon after birth [Sotiriou et al., 2002]. Our results of 30% VitC retention of controls in the depleted guinea pigs (Manuscript I) indicate that VitC in the brain is indeed retained and underlines an essential role of vitC within the brain compared to other organs. Despite preferential VitC in the brain during deficiency there was oxidative stress by increased MDA levels (Manuscript II), supporting our hypothesis of VitC deficiency-imposed redox imbalance despite a relatively high VitC concentration in the brain. We have shown high retention of VitC and elevated MDA levels even in the foetal brain (Manuscript III) supporting our hypothesis 3, that foetal brain is not protected from oxidative stress during maternal VitC deficiency. It was interesting to find elevated levels of SOD activity in the foetal brains since it was suggested by others that SOD does not increase significantly in the guinea pig foetal brain [Mishra and Delivoria-Papadopoulos, 1988a]. Together with increased SOD we have also observed that deficient foetal brain does not show the developmental trend of decline in VitC level at near-term as compensation, in contrast to the controls. Compensation mechanism was also reported earlier by us in VitC deficient weanling

guinea pig brain by an increased DNA repair activity [Lykkesfeldt et al., 2007] which however did not prevent from increased MDA levels. Reports from others have also shown compensation in the form of elevated endogenous antioxidant glutathione in VitC deficient *Gulo*^{-/-} mice brain which did not prevent increase in F2 isoprostanes [Harrison et al., 2010c]. These findings suggest that oxidative stress is caused by VitC deficiency in spite of endogenous protective mechanisms in the brain.

5.1.3 Effect of VitC deficiency induced oxidative stress is not known

Analysis of additional protein oxidation markers in our study did not support the finding by others that elevated lipid peroxidation by-products like MDA may result in oxidative modification of the proteins [Kim et al., 2000; Lung et al., 1993]. VitC *in vitro* has been indicated in inhibiting apoptosis [Guaiquil et al., 2001; Rössig et al., 2001] but we did not see significant changes in Caspase-3 mRNA or protein expression. Our results suggest that non-scorbutic VitC deficiency may not affect caspase-3 mediated apoptosis. However, it is important to consider that activation of caspase-3 independent mechanisms or necrosis may also likely accompany oxidative stress [Carmody and Cotter, 2000; Tan et al., 1998]. Based on these findings the effect of oxidative stress in VitC deficient brain is unclear and we could not associate hippocampal impairment from our previous findings to a mechanism of apoptosis. It may also be that homogenates from the whole brain instead of hippocampus could have masked the differences between control and deficient groups or the role of apoptosis is independent of hippocampal volume reduction as shown by others [Foghi and Ahmadpour, 2013].

5.1.4 Natural loss of *Gulo* makes guinea pig a valuable animal model of VitC deficiency

One of the early signs in the onset of scurvy observed in guinea pigs is the decrease in body weight [Kipp et al., 1996] which was not observed in any of the animals that were subjected to chronic non-scorbutic VitC deficiency in our studies. The animals may be prone to scurvy approximately in 2-3 weeks after following a dietary regime with VitC content of 40 mg/Kg diet or lesser [Lykkesfeldt et al., 2007] suggesting that dietary VitC manipulation in this animal model is feasible. VitC deficiency in guinea pigs may bear a resemblance to deficiency in humans due to the evolutionary loss of the *Gulo* gene and dietary VitC dependence [Yu and Schellhorn, 2013]. In case

of *Gulo*^{-/-} animal model it was suggested that the animals may have developed an adaptive mechanism by an unknown pathway [Maeda et al., 2000]. In another study, *Gulo*^{-/-} pups born to *Gulo*^{-/-} dams were reported to have increased lipid peroxidation in brain even after including sufficient VitC in their diets and despite of achieving high VitC levels similar to wild type [Harrison et al., 2008] suggesting a genotype defect in this animal model. In a later study, *Gulo*^{-/-} mice following VitC depletion were shown to have retained only 10% of brain VitC levels compared to *Gulo*^{-/-} mice fed on control diet [Harrison et al., 2010b]. Similarly other animal models with exon deletions of *Gulo*, like the osteogenic disorder shinogi (ODS) rats and the Senescence marker protein 30 (SMP30) mice, were reported to have a small flux of VitC by a minute functional activity of *Gulo* and a different pathway of VitC synthesis respectively [Kondo et al., 2006; Yu and Schellhorn, 2013]. These findings highlight differences that could exist in animal models that do not have a natural loss of *Gulo* making guinea pigs more suitable for investigating effects of VitC deficiency.

5.1.5 Implications of low VitC levels in human brain

Our current finding of increased oxidative stress as a result of non-scorbutic VitC deficiency has implications for redox-imbalance in the developing brains of infants and children. In general, newborns are at a higher risk of oxidative stress due to reduced antioxidant defence and higher risk of infections due to a developing immune system [Saugstad, 2005]. VitC in plasma has been shown to be higher in pre-term and near-term babies while other antioxidants like vitamin E and uric acid were shown to increase after term into adulthood [Lindeman et al., 1989]. This is in accordance with the finding from human foetuses and stillborn babies ranging from 12 to 38 gestational weeks in which VitC levels were reported to be increasing until late gestation [Zalani et al., 1989]. These findings suggest that VitC likely plays a vital role during development and low VitC levels may compromise normal brain development, in part due to oxidative stress.

5.1.6 Conclusions

Brain preferentially sequesters VitC at all times but not by the mechanism of an overall regulation of Svct2 expression. Non-scorbutic VitC deficiency causes oxidative stress in the brain and compensatory mechanisms does not protect the foetal brain from oxidative stress as a result of

maternal low intake of VitC. The molecular effects of VitC in the brain are obscure and need attention to discover the markers beyond scurvy to extend the line of thought from our earlier and current studies that non-scorbutic VitC deficiency disrupts normal brain development.

5.1.7 Future perspectives based on current findings

Based on our current findings on Svct2, if the preferential retention of brain VitC is not by the mechanism of Svct2 expression, then it could be due to functional regulation. Increased Svct2 activity may likely explain the sequestration of brain VitC. Alternatively ratio of the membrane localized/cytosolic expression of the transporter may be revealed by immunohistochemistry. Western blotting assessment may also give an indication of membrane localized transporters by analysis of membrane protein fractions of the brain lysates.

The effects of non-scorbutic VitC deficiency in the brain are still unclear and can only be recognized by showing more evidence from *in vivo* studies. It is important to identify the physiological consequences of oxidative stress caused by VitC deficiency because increase in oxidizing agents may affect other cellular activities [Sies, 1997]. One approach to investigate such consequences would be to investigate markers that indicate sources of biological oxidizing agents like NADPH oxidase, xanthine oxidase etc. Another approach is to investigate the cell viability by investigating molecular markers of apoptosis, necrosis and inflammation in the brain. Based on our previous finding on delayed cellular migration in the VitC deficient hippocampus [Tveden-Nyborg et al., 2012], immunohistochemistry with focus on markers of microtubule associated proteins may add support to our hypothesis of non-scorbutic VitC deficiency causing disrupted brain development.

Chapter 6

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Chapter 7

Manuscripts I, II, III

Manuscript I (In preparation)

Increased expression of vitamin C transporters in liver but not kidney and brain during deficiency

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KEYWORDS: Vitamin C, Transport, SVCT1, SVCT2, Deficiency

ABBREVIATIONS:

Asc: Ascorbate, CSF: Cerebrospinal fluid, CTRL: Control group, DEF: Deficient group, DEPL: Depleted group, DHA: Dehydroascorbate, MPA: Meta-phosphoric acid, PCR: Polymerase chain reaction, SVCT1: Sodium coupled vitamin C co-transporter 1, SVCT2: Sodium-coupled vitamin C co-transporter 2, VitC: Vitamin C.

HIGHLIGHTS

- Effects of vitamin C deficiency on the regulation of specific transporters *in vivo*
- The *Svct1* transporter in liver is increased in deficient and decreased in depleted
- No effect on *Svct1* and *Svct2* expression in kidney and brain sections
- Transport rate from plasma to CSF appears un-affected by dietary regime

ABSTRACT

The effects of vitamin C deficiency and depletion on the expression of transporters SVCT1 and SVCT2 in selected tissues and the transport from plasma to cerebrospinal fluid (CSF) was investigated in a guinea pig model. In deficient SVCT1 was increased in the liver whereas a reduced SVCT1 expression and increased *Svct2* mRNA suggests a potential shift in transporter expression in the liver of depleted animals. The plasma:CSF ratio demonstrated a constant transport irrespective of dietary regime. The study adds novel information to the complex regulation maintaining vitamin C homeostasis *in vivo* during states of deficiency.

INTRODUCTION

An association between vitamin C (vitC) deficiency and the propagation of diseases such as cardiovascular disease and metabolic syndrome has been suggested by both epidemiological studies in humans and in experimental animal models (1). VitC deficiency (defined in humans as a plasma concentration below 23 $\mu\text{mol/l}$ (2)) affects about 10% of the adult population in the industrialized world, with markedly increased prevalence in specific subgroups such as smokers and low-income citizens (3;4). As vitC is differentially distributed within the organism, there is reason to believe that a state of deficiency may impose local alterations to specific transporters. However, it is presently not known to what extent the regulation of the overall vitC homeostasis in the body is affected by various degrees of deficiency (5).

VitC displays complex dose-dependent pharmacokinetics and is subject of tight homeostatic regulation maintaining a differential tissue specific distribution (5;6). In short, absorbed vitC is found almost exclusively on its anionic reduced form, ascorbate (Asc) at physiological pH while its two-electron oxidation product, dehydroascorbic acid (DHA), is only present in negligible amounts (7). Though both passive and facilitated diffusion occur, vitC transport is primarily achieved by sodium-coupled vitamin C co-transporters SVCT1 and 2 (*SLC23A1* and *SLC23A2* respectively) governing gastrointestinal absorption, distribution to target tissue and renal reabsorption of Asc (5). The brain upholds a uniquely high Asc concentration and maintains high levels even during prolonged states of deficiency (8;9), thought to be primarily due to SVCT2-mediated transport. A pivotal role of vitC in the brain is supported by several *in vivo* findings; deficiency leading to impaired development and reduced function (10-14).

Although the tight control of vitC homeostasis and its tissue specific distribution has been recognized for years, little is known about if and how vitC deficiency affects the specific regulatory mechanisms. *In vitro* data has shown alterations of SVCT1 and 2 expression in response to changes in Asc concentration in culture medias (15;16). However, neither short- nor long-term *in vivo* studies of vitC transport to the brain have been able to demonstrate a relationship between vitC deficiency and increased SCVT2 expression, leading to speculations of possible alternative mechanisms for vitC transport (17).

Using the guinea pig as a model of diet-induced vitC deficiency, this study investigates if mRNA and protein expression of SVCT1 and 2 transporters in various tissues are affected by mild or severe chronic vitC deficiency. The study provides insight to the mechanisms underlying the regulation of overall vitC homeostasis and putative tissue-specific characteristics with emphasis on the blood-brain transport assessing vitC concentrations in plasma, cerebrospinal fluid and brain sections.

MATERIALS AND METHODS

In vivo study

The study was approved by the Danish Animal Experimentation Inspectorate under the Ministry of Justice. Twenty seven female 6-7-days old Dunkin-Hartley guinea pigs (Charles Rivers Lab, Kisslegg, Germany) were included. Upon arrival animals were equipped with a subcutaneous microchip for identification (PET-CHIP ID, e.vet®, Danworth Farm, West Sussex, UK) and randomized into three weight-stratified dietary groups (n=9/group) receiving a purified, standard guinea pig diet differing

only in vitC levels (Research Diets, New Brunswick, NJ, USA). The dietary groups consisted of a depletion group (DEPL) receiving vitC below the detectable limit of 34 mg/kg diet; a marginally deficient group (DEF:100 mg vitC/kg diet) and a control group (CTRL:2000 mg vitC/kg diet).

Animals were group housed in floor pens in an enriched environment and allowed fresh water, feed and hay *ad libitum*. By analysis, the hay did not contain vitC. Animals were tended several times daily by trained staff and body weights closely monitored.

Euthanasia

Animals were anesthetized in randomized order by inhalation of Isoflurane (Isoba Vet 100%, Intervet International, Boxmeer, The Netherlands). At the disappearance of voluntary reflexes thoracotomy was performed and an intracardial blood sample of 2-3 mL was obtained (18G hypodermic needle and 5 mL syringe flushed with 15% tripotassium-EDTA solution) (18), and the animal subsequently euthanized by exsanguination. Plasma samples were stabilized and immediately frozen on dry ice. Cerebrospinal fluid (CSF) was drained by glass-pipette through the foramen major applying a light vacuum. Samples were immediately frozen on dry ice. Tissue samples were harvested; and snap-frozen in liquid nitrogen for mRNA and protein analysis or frozen on dry ice for biochemical analysis. All tissues, CSF and plasma were placed in -80 °C for storage prior to analysis.

Ascorbate analysis

Blood samples for vitC analysis were centrifuged at 2000 x g for 5 min at 4 °C. Plasma and CSF samples were immediately mixed with an equal volume of 10% meta-phosphoric acid (MPA) containing 2 mM EDTA (Merck, Whitehouse Station, NJ, USA), centrifuged, and the supernatants were stored at -80°C until analysis (18). Tissue samples (app. 0.5 g) were homogenized in PBS,

centrifuged at 16000 x g for 1 min at 4 °C and stabilized with MPA as above. Analysis in MPA stabilized plasma, CSF and tissue homogenate were performed by high-performance liquid chromatography (HPLC) with coulometric detection as described previously (19).

Gene expression analysis

Approximately 20 mg tissue of kidney, liver, cerebellum and hippocampus were used for isolation of RNA as previously described (20). Concentration and purity of the extracted RNA was measured by spectrophotometric analysis (NanoDrop® 1000 Spectrophotometer; Thermo Scientific, Wilmington, DE, USA). cDNA synthesis was achieved by reverse transcription of 2 µg of RNA as previously described (20).

All cDNA samples were tested for DNA contamination by conventional polymerase chain reaction (PCR) before submitting to real-time quantitative PCR (Q-PCR) using an intron-spanning β -actin primer set (*F5':GTAAGGACCTCTATGCCAACAC* and *R5':ATGCCAATCTCATCTCGTTTTCT*). Only samples which did not display DNA contamination were admitted in the study. To confirm specificity of target genes PCR products were sequenced (LGC Genomics, Berlin, Germany). Real-time quantitative (Q)-PCR analysis was performed in triplicates in white 96 well plates on a LightCycler® LC480 with LightCycler® 480 SYBR Green 1 Master (all from Roche, Basel, Switzerland). Standard curves were created for individual primer-sets on calibrator dilutions, and the coherent efficiencies applied in the later expression analysis. For Q-PCR analysis, negative controls (nuclease-free water) and calibrator samples were included in all runs. Expression ratios of the two target genes *Svct1* and *Svct2* were acquired by normalization to the reference gene *Gapdh* (primer sequences are shown in table 1).

Protein extraction

Approximately 20 mg tissue (kidney, liver and cerebellum and hippocampus) was homogenized in 250 μ L cold radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% Sodium Dodecyl Sulfate [SDS]) containing Protease Inhibitor Cocktail diluted 1:100 (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged for 10 min at 1200 rpm at 4 °C. The supernatant was transferred to fresh Eppendorf tubes and frozen immediately at -80 °C. Aliquots were used for determining protein concentration using the Bradford Assay (21) and performed in triplicates (samples containing 100 mg Coomassie Brilliant Blue G-250, 50 mL 96% ethanol, 100 mL 85% phosphoric acid and 500 mL milli-Q water) at 595 nm by spectrophotometry (SpectraMax Plus 384 plate reader; Molecular Devices, Sunnyvale, CA, USA). Concentrations were calculated based on standard dilutions of Bovine Serum Albumin (Sigma-Aldrich) in Phosphate Buffered Saline (Dulbecco's PBS).

Western Blot analysis

Samples containing 40 μ g (liver, hippocampus and cerebellum) or 50 μ g (kidney) protein were adjusted with nuclease-free water to achieve a volume of 32.5 μ L, prior to adding NuPage® LDS Sample Buffer and NuPage® Sample Reducing Agent (LifeTechnologies/Invitrogen, Carlsbad, CA, USA). Following preheating (10min/70°C) samples were transferred to NuPage® Novex 4-12% Bis-Tris Midi Gels and run in duplicates at 200 Volts in NuPage® MOPS SDS Running Buffer containing NuPage® Antioxidant (Life technologies). A calibrator, a positive and a negative control was included on all gels. The separated proteins were blotted onto PVDF Transfer Membranes (TE 77 PWR semi-wet transfer unit ;GE Healthcare, NJ, USA) with NuPage® Transfer Buffer and NuPage® Antioxidant (Life technologies) at 169 mA for 45 min, followed by 2% blocking solution (1 hr in PBS, 0.1% Tween® 20 and ECL Prime™ blocking agent (GE Healthcare) followed by incubation with

primary antibody (0.4 µg/mL goat anti-SVCT1 (LOT H1111) or 1 µg/mL goat anti-SVCT2 (LOT A2313) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and 0.12 µg/mL mouse anti-actin (LOT NG1848416) (Millipore, Billerica, MA, USA,) in blocking solution at 4 °C overnight. Membranes were washed in PBS with 0.1% Tween® 20 and incubated with secondary antibody (0.1 µg/mL rabbit anti-goat for anti-SVCT1 and anti-SVCT2 and 0.04 µg/mL goat anti-mouse for anti-actin (Santa Cruz Biotechnology) in 2% blocking solution for 1 hour. Bands were visualized with Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare) using the BioSpectrum® Imaging System (UVP, Upland, CA, USA). Product size was established relative to MagicMark™ XP Western Protein Standard (Invitrogen). To determine band intensity, densitometry was performed using VisionWorks® Analysis Software (UVP) and target values normalized to the corresponding actin band and calibrator. Specificity of the primary SVCT1 and SVCT2 antibodies was confirmed by a pre-absorption test with blocking peptide in a 5:1 ratio of concentrations (Santa Cruz Biotechnology).

Statistical analyses

Data was analyzed using Statistica software (Statistica, version 9; StatSoft, Tulsa, OK, USA) applying a two-way ANOVA followed by Tukey's multiple comparisons *posthoc* test in case of significance. Homogeneity of variance was tested by Levene's test; in case of non-homogeneity, data was log-transformed prior to analysis. A *p*-value below 0.05 was considered statistically significant.

RESULTS

Bodyweights

Bodyweights were initially monitored bi-weekly and daily from day 14 due to potential risk of scurvy in the depleted group (figure 1). As expected, bodyweights of CTRL and DEF animals did not differ at euthanasia, however was significantly decreased in DEPL animals ($P<0.05$) (Table 1). When normalized to average bodyweight, brain weight was not found to be significantly different between groups.

Tissue levels of ascorbate

As expected, CTRL animals displayed a significantly higher Asc level in plasma, CSF and in all measured tissues compared to DEF and DEPL ($P<0.001$ in plasma; $P<0.0001$ liver, kidney, CSF and $P<0.05$ in brain); DEF animals were significantly lower than CTRLs and higher than DEPL on all accounts (Table 1). In CTRL animals liver and brain total Asc did not differ, however in DEF animals the brain maintained an increased Asc concentration compared to the liver and kidney, a phenomenon even more pronounced in DEPL animals. The CSF reflected the reduced availability of Asc, but interestingly, a concentration of approximately 3.5 times that of plasma in all three dietary regimes was maintained (no significant different in CSF:plasma ratio between groups). Within brain sections, the frontal cortex consistently showed higher Asc concentrations than the cerebellum in CTRL, DEF and DEPL ($P<0.001$; $P<0.01$ and $P<0.05$ respectively), displaying both an effect of diet ($P<0.001$) and an effect of tissue ($P<0.001$).

SVCT1 and SVCT2 expression analysis

In the liver, an increase in mRNA expression of *Svct1* relative to the housekeeping gene *Gapdh* was recorded in DEF animals ($P<0.05$) while a decrease in DEPL animals ($P<0.05$) compared to CTRL was observed. This pattern in SVCT1 expression was confirmed by Western blot analysis on protein

levels ($P < 0.05$). *Svct2* mRNA expression was increased in DEPL ($P < 0.05$); CTRL and DEF not differing, however a tendency to an increased expression in DEF was noted (figure 2). No detectable difference was recorded in SVCT2 protein expression between groups. No differences were detected in either protein or mRNA expression of SVTC1 or SVCT2 in any of the other investigated samples.

DISCUSSION

The present study shows an increased expression of the *Svct1* transporter in the liver during deficiency but a decrease in expression in DEPL animals on both mRNA and protein level. In contrast, expression of the *Svct2* transporter was increased in DEPL (mRNA) but not DEF. Though not supported by the Western blot analysis, a tendency towards an increased *Svct2* expression is noted. An increase in SVCT1 expression has been shown *in vitro* in human hepatic cell lines subjected to Asc depletion with increased *SVCT1* expression but no significant change in *SVCT2*, suggesting a transcriptional regulation in response to Asc depletion of culture media (22). Likewise, primary hepatocyte culture from long term vitC depleted (33 days) in *smp30/gnl*^{-/-} knock-out mice displayed increased Asc transport and an increase in both *Svct1* and an even more pronounced increase in *Svct2* (70% compared to Asc sufficient) mRNA expression (23). In agreement with our data, no effect of *in vivo* depletion on Asc transporters was detected in either kidney or brain (23). The expression pattern found in the present study indicates a differential effect of dietary regimes on the liver and suggests a *Svct1* up-regulation due to chronic but moderate deficiency, whereas a severe state of deficiency appears to shift expression from SVCT1 towards an increase in SVCT2. This is consistent with the transporter characteristics of the SVCTs.

SVCT1 is considered a high-capacity but low affinity transporter ($K_m \approx 65-252$) while SVCT2 is a low-capacity high affinity ($K_m \approx 8-69$) (5;22). Thus, in order to salvage the diminutive amounts of Asc available in plasma and limit its depletion, the liver apparently attempts to further adapt by increasing absorption through a change in its transporter profile. This is of course, provided that the recorded increase in *Svct2* mRNA expression translates to functional protein.

As expected, Asc concentrations in the brain reflected the imposed decreases in Asc supply but remained much higher than in liver and kidney for both DEF and DEPL groups. Agreeing with reports from various species including guinea pigs, Asc concentrations differed significantly between brain-sections, the frontal cortex consistently displaying higher levels than the cerebellum most likely reflecting differences in neuronal density (24-26). In the present study, no change in SVCT2 (on either mRNA or protein level) due to the imposed vitC deficiencies could be demonstrated in the investigated brain areas. This is consistent with previous findings in chronically deficient (100mg vitC/kg feed) guinea pigs compared to controls (325mg vitC/kg feed), in which no effect on *Svct1* or 2 mRNA levels was seen in liver or brain (20). In *smp30/gnl* $-/-$ knock-out mice, mRNA expression of *Svct1* and 2 in the liver was increased following vitC depletion, whereas no effect on brain was reported (23). Likewise, an increase in *Svct2* mRNA in the liver has been reported following vitC depletion in developing *gulo* $-/-$ mice, and though data points towards increased *Svct2* expression in the brain, the effect was non-significant (17).

The conundrum of the brain's ability to maintain a uniquely high Asc concentration even during severe and long-term states of deficiency without increasing levels of the specific transporter has led to speculations of a yet undiscovered mechanism of transport and regulation of vitC to the

brain (17). No significant change in plasma/CSF transport rate could be detected in the current study; the concentration in CSF remaining around 3.5 fold higher than that of plasma despite dietary regimes. Whether this transport is due to an increase in transporters located in the coroid plexus (Asc transport) and/or blood-brain-barrier or whether transporters are permanently excessively expressed to safeguard Asc supply to the brain remains to be disclosed.

CONCLUSION

We conclude that a dietary imposed chronic vitC deficiency in guinea pigs leads to changes in the SVCT1 and SVCT2 expression in the liver but not in brain or kidney. With respect to the hypothesis of the putative modulation of brain transport during deficiency, the finding of a constant vitC concentration ratio from plasma to CSF regardless of dietary status reveals that this interface is not the site of modulation.

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Table 1: Primer sequences of housekeeping and target genes.

Gene	Primer sequence (5'-3')	Product size	NCBI Acc. No.
<i>Svct1</i> ⁽²⁰⁾	F: TCCGACAGATTATGGCTTCC	211	AF410935
	R: GCACAAGCATAGTAATCACCGA		
<i>Svct2</i> ⁽²⁷⁾	F: GTCCATCGGTGACTACTA	114	AF411585
	R: ATGCCATCAAGAACACAGGA		
<i>Gapdh</i> ⁽²⁰⁾	F: GCACCGTCAAGGCTGAGAAT	227	DQ403052
	R: CATCACGAACATAGGGGCATC		

Table 2: Vitamin C levels in selected tissues

	Control (CTRL) 2000 mg/kg feed n=9	Deficient (DEF) 100 mg/kg feed n=9	Depleted (DEPL) 0 mg/kg feed n=9
Plasma total ascorbate ($\mu\text{mol/L}$)	73.5 ± 31.5^a	$5.08 \pm 2.8^{b**}$	$0.99 \pm 0.1^{c**}$
CSF total ascorbate ($\mu\text{mol/L}$)	247.9 ± 30.8^a	$16.6 \pm 8.5^{b***}$	$3.3 \pm 1.3^{c***}$
Liver total ascorbate (nmol/g tissue)	1762.8 ± 554.4^a	$269.4 \pm 131^{b**}$	$26.8 \pm 2.9^{c**}$
Kidney total ascorbate (nmol/g tissue)	757.0 ± 170.6^a	$166.6 \pm 78.8^{b**}$	$13.8 \pm 4.4^{c**}$
Frontal cortex total ascorbate (nmol/g tissue)	$1654.6 \pm 156.2^{a,****}$	$826.5 \pm 125.0^{b*,***}$	$518.5 \pm 22.4^{c*,\#}$
Cerebellum total ascorbate (nmol/g tissue)	1013.6 ± 333.1^a	$584.9 \pm 161.1^{b*}$	$313.2 \pm 64.0^{c*}$
Body weight at euthanasia (g)	312 ± 21.94^a	313 ± 27.59^a	$275 \pm 78^{b*}$
Brain weight normalized to BW	3.4 ± 0.32	3.5 ± 0.41	3.1 ± 0.30

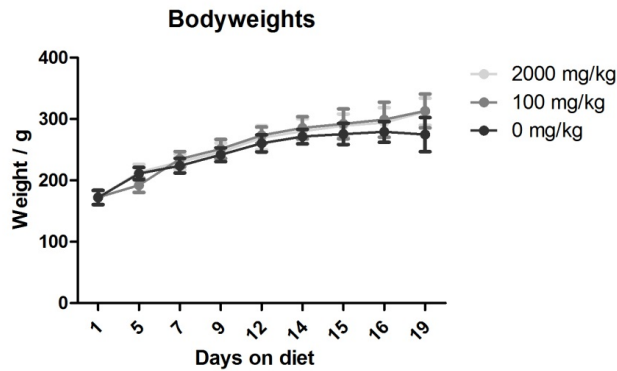
Results are presented as means \pm SD. Dissimilar superscripts denotes values of significant difference.

#: Difference in ascorbate concentration in brain sections (frontal versus cerebellum) within the dietary group. I.e. Asc in frontal cortex is significantly higher than in cerebellum, $P < 0.0001$ (CTRL).

* $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$.

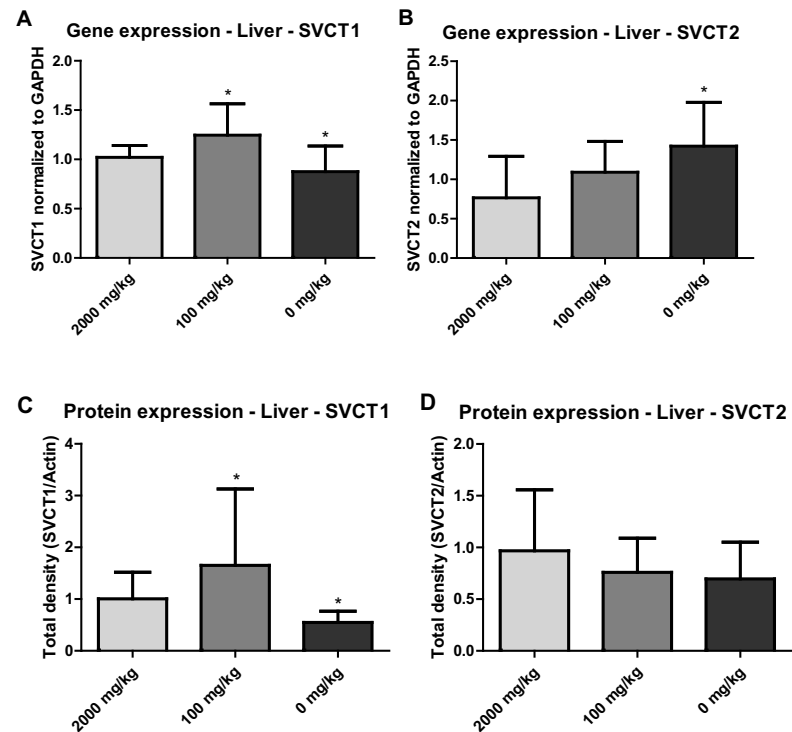
FIGURE LEGENDS

Figure 1: Recorded bodyweights in the dietary groups during the study period.



As weight loss is an initial sign of emerging scurvy (9) and a humane end-point of this study, bodyweights were monitored closely. As expected weight loss appeared in the DEPL group ($P<0.05$), after which all animals were euthanized before any clinical symptoms of scurvy could be detected.

Figure 2: Effect of vitC deficiency on the expression of SVCT1 and SVCT2 in the liver.



On both mRNA and protein level (panel A and C) SVCT1 expression was increased in DEF and decreased in DEPL. An increase in *Svct2* mRNA expression was found in DEPL (panel B) and though this also appears to be present in DEF a difference to CTRL was not significant. No effect on SVCT2 protein was found between groups (panel D). *P<0.05.

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Chronic vitamin C deficiency promotes redox imbalance in the brain but does not alter sodium-dependent vitamin C transporter 2 expression

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Abstract

Background: Vitamin C (VitC) has several roles in the brain acting both as a specific and non-specific antioxidant. The brain upholds a very high VitC concentration and is able to preferentially retain VitC even during deficiency. The accumulation of brain VitC levels much higher than in blood is primarily achieved by the sodium dependent VitC transporter (SVCT2). This study investigated the effects of chronic pre-and postnatal VitC deficiency as well as the effects of postnatal VitC repletion, on brain SVCT2 expression and markers of oxidative stress in young guinea pigs.

Results: Biochemical analyses demonstrated significantly decreased total VitC and an increased percentage of dehydroascorbic acid as well as increased lipid oxidation (malondialdehyde) in the brains of VitC deficient animals ($p < 0.0001$) compared to controls. VitC repleted animals were not significantly different from controls. No significant changes were detected in either gene or protein expression of SVCT2 between groups or brain regions.

Conclusion: In conclusion, chronic pre-and postnatal VitC deficiency increased brain redox imbalance but did not increase SVCT2 expression. Postnatally repleted pups did not display the redox imbalance observed in deficient counterparts. Our findings show potential implications for VitC deficiency induced negative effects of redox imbalance in the brain and provide novel insight to the regulation of VitC in the brain during deficiency.

Key Words:

- **Vitamin C deficiency**
- **SVCT2**
- **Redox imbalance**
- **Oxidative stress**

BACKGROUND

Vitamin C (VitC) has multiple roles in the brain acting both as a non-specific antioxidant [1, 2] and as a co-factor of enzymatic reactions including collagen- and catecholamine synthesis [3, 4] as well as being involved in neuronal glutamate re-uptake [3]. VitC is preferentially retained in the brain relative to other organs even during states of deficiency, emphasizing the prominence of this essential micronutrient in the brain [5, 6]. Due to a high level of cellular metabolic activity and a high content of lipids, the brain, and particularly the growing brain, is prone to oxidative stress and lipid peroxidation [7]. Oxidative stress is defined as an imbalance in redox-homeostasis, e.g., between free radicals and the coherent protection by anti-oxidants [8]. Hence, oxidative stress is propagated when VitC supply is insufficient [9, 10], hereby leading to imbalance in redox homeostasis and subsequent progressive cellular damage. Under such circumstances, lipid peroxidation has been a principal biomarker to assess oxidative damage in the brain and has been suggested to be associated with cognitive impairment and neurodegenerative diseases [11, 12].

VitC enters the brain primarily in its reduced form, ascorbate (ASC) [13]. From the blood stream ASC is transported against a concentration gradient into brain through the choroid plexus to the cerebrospinal fluid (CSF) and from here reaches the brain to achieve homeostasis [3]. The majority of VitC transport to the brain is accomplished through an active transport by the sodium dependent vitamin C transporter (SVCT2), allowing VitC levels to reach concentrations 10 fold higher than that of the blood [14, 15]. Mice lacking this transporter have been shown to die immediately after birth, displaying respiratory failure and haemorrhage in the brain [16, 17]. A study with *Svct2*^{+/-} mice compared to wild type demonstrated that VitC levels in brain cortex were genotype dependant with higher VitC content in mice homozygous for *Svct2*^(+/+) compared to heterozygous counterparts [17].

Within the brain differential VitC retention in specific brain regions has been reported [18]. Dietary intervention studies have shown increases in *Svct2* mRNA expression in the liver, both *in vitro* and *in vivo*, but not in the brain following VitC deficiency in mice unable to synthesize VitC (*Gulo*^{-/-}) [19, 20]. However analysis of the *in vivo* SVCT2 expression in the cerebellum disclosed an increase in protein levels but not mRNA following VitC deficiency in adult *Gulo*^{-/+} mice. Although the increase was not significant, it showed an apparent response to reductions in VitC supply unlike in cortex of the same group, which was suggestive of tissue dependent regulation of SVCT2 [20, 21].

Like humans and primates, guinea pigs cannot synthesize VitC due to mutation in the L-gulonolactone-oxidase gene and therefore VitC must be supplied in the diet to prevent the development of scurvy [22]. Hence, the guinea pig is considered a valuable model to investigate effects of VitC deficiency. We have previously shown that VitC deficiency in newly weaned guinea pigs elevates DNA repair and oxidative stress [23], and is associated with an impairment of spatial memory and reduction of hippocampal neurons in young guinea pigs with low levels of VitC [24]. In a recent study, we have shown a persistent hippocampal volume reduction in prenatally deficient animals regardless of postnatal VitC repletion [25].

In this study, we investigated if a chronic pre- and postnatal VitC deficiency in guinea pigs leads to postnatal (day 70, P70) lipid peroxidation in the brain, if prenatal damage persists following postnatal repletion, and if differences observed associate to gene and protein expression of SVCT2 transporter in three brain regions, hippocampus (HP), cerebellum (BC) and brain frontal cortex (BFC). We show that chronic pre- and postnatal VitC deficiency leads to redox imbalance by increase in ascorbate oxidation and malondialdehyde (MDA) in young guinea pig brains, however this does not result in changes in SVCT2 expression.

RESULTS

Biochemical analyses

As expected, VitC deficiency was reflected in the VitC concentrations in brain and plasma with lower levels in DEF animals and the %DHA in plasma and brain showing elevated oxidation of the VitC pool ($p < 0.0001$ for both ; Table 2). No significant differences were found between VitC levels in CTRL and REPL groups demonstrating that REPL pups reached CTRL-status for VitC (Table 2). VitC status significantly affected brain MDA concentrations ($p < 0.0001$) with the DEF group displaying higher lipid oxidation compared to CTRL or REPL ($p < 0.0001$; Table 2). No significant difference in MDA level was found between CTRL and REPL groups at day 70. In contrast to MDA levels, dietary VitC did not affect brain glutathione levels significantly.

Svct2 expression

The PCR analysis of *Svct2* expression from HP displayed a tendency to increase in DEF animals, but this was not significant (Figure 1A). No significant differences were observed in either BC or BFC regions between the three groups (Figures 1B & 1C). Western blot was performed to investigate if VitC deficiency affected SVCT2 protein levels from the three brain regions. Samples resulted in a doublet that may correspond to glycosylated and non-glycosylated forms (bands above 60 kDa and ~70kDa) (Figure 2A), as has been previously reported [31, 32]. Incubation with antibody specific blocking peptide successfully prevented both bands (Figure 2B), confirming specificity of the SVCT2 antibody in both guinea pig and mouse (positive control) lysates (Figures 2A & 2B). A few brain lysates gave rise to an additional 80 kDa band following anti-SVCT2 blots. This was detected even after pre-

absorption with blocking peptide, confirming it to be a non-specific band (Figure 2B). Although we did not quantify band density between the three regions of the brain, hippocampus samples showed intense bands compared to the samples from the other two brain regions, with the faintest bands pertaining to samples from BFC. No significant changes were found between the three groups within the measured brain regions (Figure 3) suggesting that SVCT2 expression is not induced by the dietary regimes applied in this study.

DISCUSSION

In the present study, we wanted to assess the effect of chronic pre- and postnatal VitC deficiency on oxidative stress markers and VitC transporter expression in the brain of young guinea pigs (P70), and if prenatal effects persisted after postnatal repletion. As expected, deficient animals had significantly lower levels of VitC in plasma while VitC levels did not differ between CTRL and REPL groups in spite of the difference in VitC status of their mothers (data not shown) [33]. DEF animals also showed increased oxidative stress measured by ascorbate oxidation (%DHA) and MDA. Other studies have reported MDA as a marker of lipid oxidation in the brain and increases in %DHA is associated to an enhance interaction between VitC and oxidants. The findings of both lipid oxidation and the promotion of VitC oxidation suggests increased levels of oxidative stress in DEF animals, which could result in disruption of the established metabolic pathways in the brain.

Low levels of VitC has been shown to increase GSH synthesis in brain of *Gulo*^{-/-} mice [34] and VitC has also been suggested to modulate GSH regulation in human erythrocytes [35]. Our data did not show any significant changes in brain total GSH suggesting that the chronically low levels of VitC in this study may still be sufficient to spare GSH in the brain. Moreover, VitC is capable of maintaining other anti-oxidants such as vitamin E in a reduced

state [36]. However, previous investigations in VitC depleted guinea pigs have shown that despite the positive retainment of VitC in the brain during acute VitC deficiency, oxidative stress is not prevented and may have detrimental consequences for cellular function and survival [23].

No significant differences were found between CTRL or REPL groups for any of the measured parameters. The absence of a detectable difference between CTRL and REPL at P70 in both biochemical and molecular markers is likely due to VitC levels being restored at an earlier time point after repletion. We have recently reported that overall hippocampal volume was significantly reduced in prenatally deficient guinea pigs and persisted despite postnatal repletion [25]. This difference could be due to the hippocampal impairment occurring at a much earlier time-point (i.e. prenatally), as opposed to the currently presented data which investigates animals at postnatal day 70, thus much later than when the VitC deficiency was initially imposed. However, we have recently investigated the effects of prolonged maternal VitC deficiency in neonate (P7) guinea offspring [33]. Brain VitC levels in the neonate VitC deficient pups were 60% lower than the control group but there was no difference in ascorbate oxidation ratio between deficient and control pups. No effect of maternal VitC deficiency on either MDA or F₂-isoprostanes in brain tissue of pups was found [33]. As the data is exclusively obtained in postnatal pups conclusions cannot be made concerning a potential VitC deficiency induced increase in fetal oxidative stress. However, the findings suggest that the increase in lipid oxidation and %DHA in the P70 guinea pigs included in the present study has occurred primarily as a result of postnatal deficiency.

In the brain, SVCT2 is the only known specific transporter that transports ASC into neurons [37, 38]. Although mRNA expression of this transporter tended to increase in HP samples of the DEF group, the groups were not significantly different and this tendency was not confirmed by western blot. No significant differences in either gene or protein expression of

SVCT2 was found in BFC or BC samples in coherence with reports from studies in mice [20, 21]. Western blots with anti-SVCT2 showed double bands in all samples as has previously been reported [31, 32]. Decreasing the total protein load [39], on the gels did not resolve the problem. Previous studies reported a range from 50 kDa to 65kDa for SVCT2 in western blots from various tissues and species, and this is attributed to variable glycosylation and species specificity [39-42]. Although we did not find any significant changes in SVCT2 protein expression, we cannot rule out the possibility of specific regional increases within the investigated brain areas. It can also be speculated that SVCT2 levels may have increased during perinatal life only to have dropped by P70. Differences in SVCT2 expression during pre- and postnatal life has been shown in mice documenting a developmental regulation of this transporter [20, 43]. However, no significant effect of postnatal VitC deficiency in *Gulo*^(-/-) mice on SVCT2 expression in the brain was reported although a tendency of an upregulation of SVCT2 protein in cerebellum of VitC deficient pups was proposed, indicating a regional-specific SVCT2 regulation [20].

Our results are in accordance with dietary intervention studies of VitC deficiency in *Gulo*^(-/-) mice, in which low levels of VitC resulted in increase in oxidative stress markers [18, 34, 44]. However, in these studies maternal environment was protected by supplementing the *Gulo*^(-/-) dams to meet the demands of pregnancy. VitC deficiency was thus imposed postnatally to the newborn pups. Maternal environment of low VitC group in our present study is chronically low by gestational day 40 (data not shown) [33]. Thus, a consequence of a combined pre- and postnatal VitC deficiency is assessed as well as the effect of re-introducing high levels of VitC immediately after birth.

VitC reaches the central nervous system through the choroid plexus, thus a differential regulation of the transporter particularly in this area remains a possibility. A study of regional brain ischemia in rats showed loss of *svct2* mRNA in the ischemic core followed by an

increase in the peri-infarct regions at 22h of reperfusion[45]. Similarly, the SVCT2 protein in brain was shown to increase on 2d and 5d following ischemia in mice and was also found to be increased around the ischemic core in areas where it would not usually be detected [46]. It should be noted, that the above studies have reported a difference in expression of this transporter two or five days after inducing ischemia, representing a short term effect as opposed to the chronic regime applied in our study.

Another possibility for not finding significant differences in brain SVCT2 expression corresponding to prolonged VitC deficiency would be because of tight regulation of the transporter and post-translational modifications. SVCT2 is supposedly glycosylated in-vivo to maintain its functionality [38] and mutations in glycosylation sites of human SVCT2 significantly decreased VitC uptake in HepG2 cells [47]. Some *in vitro* studies have suggested that SVCT2 is regulated depending on the redox status of the cells showing an up-regulation of the transporter in the presence of oxidants [32, 48]. Our findings propose that a chronic low VitC level is indeed associated with an increase in lipid peroxidation but does not increase SVCT2 in the measured brain regions. This could be due to variable stringency with which different factors can control the expression of SVCT2 in tissues.

CONCLUSIONS

In conclusion, chronic VitC deficiency during early life promoted postnatal redox imbalance in the brain, which was not observed in repleted animals. However, no association between dietary VitC and SVCT2 expression on either mRNA or protein level in the brain was observed. Our results suggest that modulation of SVCT2 expression within specific brain regions is not a potential mechanism to compensate for a chronic state of VitC deficiency in the brains of young guinea pigs.

METHODS

Animal experimentation

The study was approved by Danish Animal Experimentation Inspectorate and in accordance with EU Directive 2010/63/EU for animal experiments. Animals were obtained as subsets in a large *in vivo* study [25]. Eighty pregnant Dunkin Hartley guinea pigs at gestation day 18 (Charles Rivers Lab, Kieslegg, Germany) were equipped with subcutaneous (s.c.) microchips for identification (PET-CHIP ID, e.vet[®], Danworth farm, West Sussex, UK), and randomized into weight stratified dietary groups receiving sufficient (900 mg, n=30) or deficient (100 mg, n=50) levels of VitC per kg diet (quality controlled diets by Special Diets Services, SDS, Witham, England). We have previously shown that the dose of 100 mg VitC/kg feed results in a non-scorbutic deficiency status in guinea pigs [24]. In this study, thirty female pups (n=30) were included, forming three dietary groups differing only in VitC content of the feed: control (pre-/postnatally sufficient, CTRL, 900/750 mg vitC/kg diet), deficient (pre-/postnatally deficient, DEF, 100/100 mg vitC/kg diet) or repleted (prenatally deficient/postnatally sufficient, REPL, 100/750 mg vitC/kg diet). The animals were housed in floor pens and allowed feed, hay and water *ad libitum*. They were weighed at least once a week. VitC status was verified by blood sampling (~ 300 µl) from *v. saphena* at its superficial course on tibia around postnatal day (P) 35 (data not shown).

Euthanasia

Animals were anesthetized by inhalation with isoflurane (Isoba Vet 100%, Intervet International, Boxmeer, The Netherlands). After disappearance of voluntary reflexes (palpebral and interdigital), thoracotomy was performed and an intracardial blood sample was obtained using a 5 ml syringe and 18G-needle previously flushed with 15% tripotassium-

EDTA. Animals were sacrificed by exsanguination and subsequent decapitation. Blood samples were immediately centrifuged and stabilized. Brains were excised and weighed before sectioning through the cerebral longitudinal fissure. One hemisphere (randomized left/right) was subsequently intended for biochemical, gene and protein expression analysis; for gene and protein analysis HP, BC and BFC were isolated and snap-frozen in liquid nitrogen. Remaining brain tissue was frozen on dry ice for biochemical analysis. All the excised tissues were stored at -80°C until use. The paired hemisphere was stored for hippocampal volume assessment [25].

Biochemistry Analyses

Ascorbate and dehydroascorbic acid (DHA), the reduced and oxidized forms of VitC, respectively, in plasma and brain as well as malondialdehyde (MDA) and glutathione in brain were analyzed as described previously [26-28].

RNA extraction and RT-PCR

RNA isolation was performed as described previously [29]. Briefly, approximately 25 mg of each of BC, BFC and HP tissues were homogenized in trizol (InVitrogen, Merelbeke, Belgium) and precipitated with chloroform (Sigma, Steinheim, Germany) and isopropanol (Merck, Darmstadt, Germany). The resulting RNA was purified using spin columns according to manufacturer's instructions (SV Total RNA Isolation System, Promega, Madison, WI, USA) and was eluted with 50 µl nuclease free water. The purity of RNA was determined by spectrometry (Nanodrop 2000; Thermo Scientific, Wilmington, DE, USA) with absorbance ratios A260/A280 and A260/A230. RT-PCR was performed with 2 µg of RNA in duplicates yielding a total volume of 50 µl cDNA for each sample (MmLV RT enzyme, 5 x MmLV buffer and RNasin (Promega)); 10 mM dNTPs and Oligo (dT) primers (60 µg/120 µl)

(Fermentas GmbH, St Leon Roth, Germany); Random hexamer primer (2 µg/µl) (GE Healthcare, Uppsala, Sweden).

Gene expression analysis

All cDNA samples were tested for DNA contamination with intron-spanning *beta-actin* primers (Table 1) prior to real time quantitative PCR (Q-PCR) and only included if negative for contamination. PCR products of included genes were confirmed by electrophoresis in 2% agarose gel, followed by PCR clean-up (PCR Clean Up System; Promega, Sweden) and subsequent sequencing of PCR products (LGC genomics, Berlin, Germany).

For Q-PCR analysis, efficiency generated from specific standard curves was applied to each run. Q-PCR was conducted (SYBR Green I master LC480 and LC480, Roche, Basel, Switzerland) in 96-well white plates (Roche, Mannheim, Germany) with triplicates of all samples (in dilution 1:5), nuclease free water as negative control and calibrator as positive control. Target gene expression analysis of *Svct2* [30] from the three different brain samples was done by normalizing to the reference gene, *s18* (ribosomal protein S18). Primer sequences are displayed in Table 1.

Protein extraction and Western blot

Approximately 20 mg of brain tissue was homogenized in 250 µl ice cold radio-immuno-precipitation assay (RIPA) buffer with protease inhibitors (150mM sodium chloride, 1 % Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50mM Tris, pH 8, 1:100 sigma complete protease inhibitor cocktail) and centrifuged at 12000 rpm for 10 minutes at 4°C. The resulting supernatant was transferred into aliquots and protein estimation was done using Bradford assay (Coomassie brilliant blue G-250 (Fluka, Damstadt, Germany), ethanol 96 % (Danish distillers, Roskilde, Denmark), phosphoric acid 85% (Merck), 1 mg/ml bovine albumin (Sigma) with brain lysates diluted in PBS (Dulbecco's, pH 7.4) in triplicates

at 595 nm on a SpectraMax Plus 384 UV/VIS plate reader (Molecular Devices Inc., CA, USA).

Samples of approximately 30 µg of protein diluted with loading buffer and sample reducing agent (Invitrogen NuPAGE 4X LDS sample buffer, Invitrogen NuPAGE 10X sample reducing agent) were heated at 70°C for 10 minutes and were loaded onto pre-cast polyacrylamide gels (Invitrogen NuPAGE 4-12% Bis-Tris gels). All samples were run in duplicates and electrophoresis proteins were transferred to PVDF membranes (GE Health Care, Sarl Fribourg, Switzerland) in a semi wet blot chamber (TE 77PWR, Amersham Biosciences) at 169 mA for 45 minutes. Membranes were blocked for 1 hour in 2 % blocking buffer (Amersham ECL Prime Blocking Agent) diluted in wash buffer (1XPBS and 0.1 % Tween) and incubated with primary antibody, SVCT2 (1:200, anti-goat IgG, Sc-9926, Santa Cruz Biotechnology, CA, U.S) or Actin (1:20000, Mouse Anti Actin IgG₁, Millipore, Temecula, CA, U.S) in blocking buffer at 4°C overnight. Specificity of the antibody was tested by a pre-absorption test with blocking peptide for anti-SVCT2 (SC9926-P, Santa Cruz Biotechnology). After washing the membrane, following secondary antibody incubation in 2 % blocking buffer (1:4000, anti-goat IgG-HRP or 1:10,000, anti-mouse IgG-HRP, both from Santa Cruz Biotechnology) for one hour, the bands were visualized by enhanced chemiluminescence (Amersham ECL Prime Western Blotting Reagent, UVP Biospectrum imaging system). Band location was identified by a western protein standard (Magic Mark™ XP Western Protein Standard). Densitometry was performed with UVP Life Science Series Software. SVCT2 band intensities were initially normalized to respective actin bands. Normalization between blots was done to control sample that was also run in duplicates at the same position on all gels, as an internal control. SVCT2 expression was finally normalized relative to corresponding internal control.

Statistics

Differences in biochemistry, mRNA and protein expression were analysed by using one-way ANOVA followed by Tukey's multiple comparisons posthoc test in case of statistical significance. Variance homogeneity was analysed by Levine's test for equal variance and data transformation was done when Levine's test for equal variance was significant ($p < 0.05$). All analyses were conducted using SAS/JMP version 8.0.

COMPETING INTERESTS The authors declare that they have no competing interests.

AUTHORS CONTRIBUTIONS

JL, PTN and JGS planned the study; JGS, MDP and PTN performed the in vivo experiment, MDP and JL performed the data analysis; MDP, PTN, JGS and JL wrote the paper.

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Table 1 Primers for PCR and Q-PCR

Gene	Primer Sequence	Product size (bp)	NCBI Accession No.
<i>Beta-actin</i>	(F): gtaaggacctctatgccaacaca (R): atgccaatctcatctcgttttct	346	[GenBank:AF508792]
<i>s18</i>	(F): atgtggtgttgaggaaagcag (R): gcttgttgcagaccgttg	195	[GenBank:XM_003473925.1]
<i>Svct2</i>	(F): gtccatcggtgactacta (R): atgccatcaagaacacagga	114	[GenBank:AF411585]

All primer sequences are presented in 5'-3' direction (F): forward primer, (R): reverse primer. Sequence analysis confirmed the sequences similarity with the presented NCBI GenBank Accession numbers.

Table 2 Biochemical results from plasma and brain

	CTRL	REPL	DEF	Effect
Plasma VitC (nmol/ml)	51.8 ± 22.4 ^a	57.3 ± 13.6 ^a	4.0 ± 3.2 ^b	***
Plasma DHA% (% of total VitC)	10.2 ± 4.9 ^a	7.7 ± 2.2 ^a	19.1 ± 3.8 ^b	***
Brain VitC (nmol/g tissue)	1399 ± 143 ^a	1498 ± 42 ^a	495 ± 252 ^b	***
Brain DHA% (% of total VitC)	4.5 ± 2.8 ^a	3.1 ± 2.2 ^a	9.5 ± 4.6 ^b	***
Brain MDA (nmol/g tissue)	313 ± 124 ^a	258 ± 58.9 ^a	476 ± 106 ^b	***
Brain GSH (nmol/g tissue)	1348 ± 112 ^a	1407 ± 44.6 ^a	1341 ± 101.0 ^a	

Biochemistry results of CTRL, REPL and DEF guinea pigs from brain and plasma following a prenatal VitC deficiency and subsequent two months postnatal repletion or deficiency, compared to pre-and postnatally sufficient controls. Effect of VitC diet between three groups by one way ANOVA marked *** $p < 0.0001$. All values are presented as means ± SD. Values with different superscript letters are significantly different.

Figure 1 Quantitative PCR analysis of *Svct2* mRNA expression in brain.

Quantitative PCR analysis of *svct2* from three brain regions of guinea pigs between CTRL, REPL and DEF groups ; Expressed values are mean of normalized ratio of *svct2* to the reference gene *s18* \pm SD ; target gene expression in HP (A), BC (B) and BFC (C), n=10 for each group. Effect of diet between the three groups was assessed by one way ANOVA ($p>0.05$).

Figure 2 Specificity of anti-SVCT2 in western blot.

(A) Specificity of anti-SVCT2 without pre-absorption of antibody with blocking peptide in guinea pig (Gp) brain lysates seen as a doublet above 60kDa and in mouse (M) brain lysates as a single band at 60 kDa; (B) Show brain lysates of Gp and M with pre-absorption of antibody with blocking peptide. Block arrow show the non-specific band detected in western blots from a few guinea pig brain lysates.

Western blot standard ladder (Std) and band lengths are displayed for both blots.

Figure 3 Western blot analysis of SVCT2 protein in brain.

Densitometry analysis of western blot analysis of SVCT2 protein levels from three brain regions of guinea pigs HP (A), BC (B) and BFC (C).; CTRL: control group, REPL: repleted group and DEF: deficient group. Values are displayed as mean of normalized ratio to actin \pm SD.

Figure 1

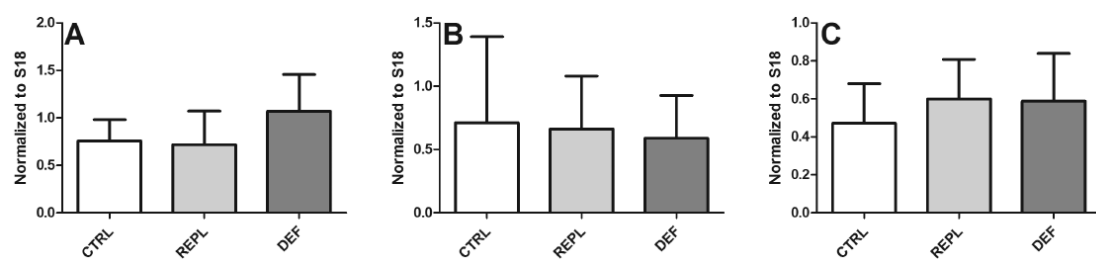


Figure 2

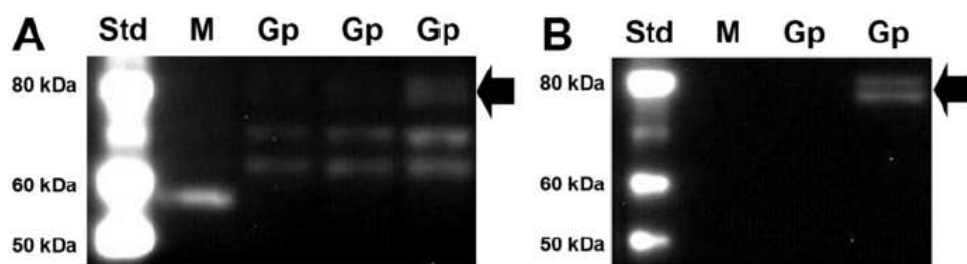
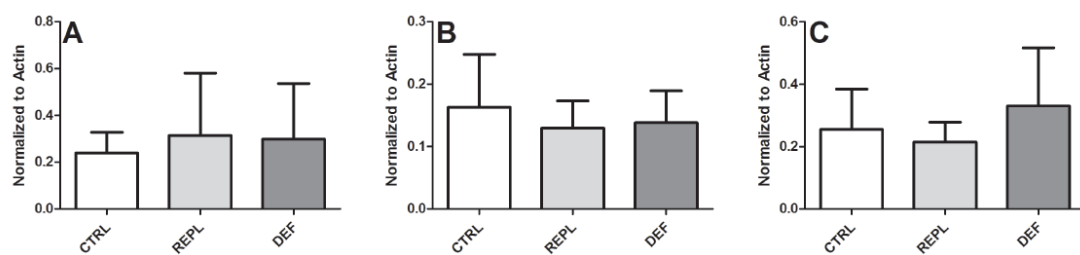


Figure 3



Manuscript III Published in Redox Biology

Prenatal vitamin C deficiency results in differential levels of oxidative stress during late gestation in foetal guinea pig brains

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ABSTRACT

Antioxidant defences are comparatively low during foetal development making the brain particularly susceptible to oxidative stress during antioxidant deficiencies. The brain is one of the organs containing the highest concentration of vitamin C (VitC) and VitC deficiency during foetal development may place the brain at risk of redox status imbalance. In the present study, we investigated the developmental pattern and effect of VitC deficiency on antioxidants, vitamin E and superoxide dismutase (SOD), assessed oxidative damage by measuring malondialdehyde (MDA), hydroxynonenal (HNE) and nitrotyrosine (NT) and analysed gene and protein expression of apoptosis marker caspase-3 in the guinea pig foetal brain at two gestational (GD) time points, GD 45/pre-term and GD 56/near term following either a VitC sufficient (CTRL) or deficient (DEF) maternal dietary regime. We show that except for SOD, antioxidants and oxidative damage markers are differentially expressed between the two GDs, with high VitC ($p < 0.0001$), NT modified proteins ($p < 0.0001$) and active caspase-3 levels ($p < 0.05$) at pre-term and high vitamin E levels ($p < 0.0001$), HNE ($p < 0.0001$) and MDA ($p < 0.0001$) at near term. VitC deficiency significantly increased SOD activity ($p < 0.0001$) compared to CTRLs at both GDs indicating a compensatory response, however, low levels of VitC significantly elevated MDA levels ($p < 0.05$) in DEF at near term. Our results show a differential regulation of the investigated markers during late gestation and suggest that immature brains are susceptible to oxidative stress due to prenatal vitC deficiency in spite of an induction of protective adaptation mechanisms.

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Introduction

The developing brain has a high metabolic activity making it vulnerable to oxidizing agents such as free radicals [55], a situation that is further exacerbated by a yet immature scavenging antioxidant system in early life [33,48]. Although antioxidant defence mechanisms in guinea pig brains during foetal development have been investigated [36], the manifestation of macromolecular modifications due to lipid peroxidation or by similar oxidizing agents and cell death in the near term brain is unknown. It is also not known if the pertinent oxidative changes during development

are likely to increase in the brain when deprived of optimal antioxidant levels.

Guinea pigs are precocial rodents with a gestation length of approximately 66 days with the peak of exponential brain growth ('the brain growth spurt') being reached about 15 days before birth (i.e. around gestation day (GD) 50) [12]. Interference with optimal requirements for the foetus may impose several negative effects on developmental outcome such as lower body weight [11] and in the brain lead to reduction of cells in hippocampal morphogenesis [4] and cognitive alterations as observed in rats [38]. Oxidative stress caused by deficiency in antioxidants like glutathione in the developing brain has been shown to negatively affect mitochondria [21] and also to result in an age-dependent neurodegeneration in the hippocampus [1].

Activity of antioxidant enzymes in guinea pigs such as superoxide dismutase, catalase, glutathione peroxidase (GPx) and glutathione reductase have been demonstrated to remain constant from GD 30 to 45 and to increase from GD 45 to GD 60, with the exception of superoxide dismutase (SOD) that remained constant throughout the GD 30–60 period [36]. In rats, postnatal GPx activity in the brain has been found to remain constant from birth

Abbreviations: ¹VitC, vitamin C; GD, gestational day; GPx, glutathione peroxidase; MDA, malondialdehyde; NT, nitrotyrosine; HNE, hydroxynonenal; SOD, superoxide dismutase; PCR, polymerase chain reaction; PFA, paraformaldehyde; s18, ribosomal protein 18S; CTRL, control; DEF, deficient

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whereas the activity of cytoplasmic SOD increases from birth until 60 days after birth in rats [34]. Analysis of antioxidant protein levels in developing mice brains from embryonic day 18 to postnatal day 21 has shown that total SOD activity, catalase and GPx activity increases suggesting an increase in antioxidants in perinatal and neonatal brains relative to foetal development [25]. Thus, despite inter-species differences and differences in degree of postnatal development (i.e. altricial vs. precocial species), there seems to be a general trend of increasing antioxidant capacity in the brain during the course of development. Hence, a decrease in antioxidant capacity could potentially disturb the redox homeostasis in the brain leading to negative consequences on development.

Vitamin C (VitC) deficiency in humans has been associated with increases in premature births and predisposing newborns' to oxidative stress [39,44]. VitC is one of the primary antioxidants in the brain and selectively accumulates in high amounts also during foetal development [14,26,33]. Interruption of VitC transport to the brain in the *svct2*^{-/-} mouse is detrimental to perinatal survival [13,50], underlining the pivotal role of VitC in the developing central nervous system. Due to its one electron reduction potential, VitC effectively inhibits lipid peroxidation and scavenges several reactive oxidizing compounds such as superoxide, hydrogen peroxide and hydroxyl radicals as well as enabling the recycling of other antioxidant compounds such as vitamin E [7,20,30,49]. In guinea pigs, VitC deficiency during both pre- and postnatal development has been linked to deviations in hippocampal development [51,52]. Alteration of apoptotic mechanisms due to redox imbalance in the foetal brain has also been reported in several studies, however, the underlying mechanism and potential cause vs consequence remains to be disclosed [19,43].

Like humans, guinea pigs cannot synthesize VitC due to a non-functional gulonolactone oxidase (*Gulo*) gene [40] and therefore rely on an adequate dietary supply. The present study investigated the developmental course of and potential effects of VitC deficiency on markers of antioxidants, redox imbalance and apoptosis signalling in foetal guinea pig brains at two different gestational time points at which brain growth is at a peak, GD 45/pre-term and GD 56/near term.

Materials and methods

Animal experiment

The animal study adheres to the guidelines of EU Directive 2010/63/EU and was approved by Danish Animal Experimentation Inspectorate. Twenty pregnant guinea pigs between GD 6–10 were obtained from Charles Rivers Lab, Killeslegg, Germany. The animals were microchipped subcutaneously (PET-CHIP ID, Danworth farm, West Sussex, UK) and randomized according to GD and body weight into two groups receiving diets only differing in VitC content (specialized diets sniff, GmbH), Control: CTRL (900 mg/kg diet, *n* = 10) and Deficient: DEF (100 mg/kg diet, *n* = 10). It has previously been shown by us that the 100 mg VitC diet in guinea pigs results in non-scorbutic VitC deficiency [32,53]. Each of the groups was further randomized to having caesarian section (followed by euthanasia) performed at GD 45 or GD 56. The animals were housed in floor pens with straw bedding and feed, hay and water were provided ad libitum. They were weighed once every week and blood was sampled (~300 µl) once in every two weeks from *v. saphena* at its superficial course on tibia to verify VitC status (data not shown).

At euthanasia three dams, one from CTRL group and two from DEF group were found not to have conceived. Necropsy revealed no signs of underlying disease and the animals were excluded from the study.

Euthanasia

Caesarean section was conducted on dams at GD 45 or GD 56. Ten to fifteen minutes prior to anaesthesia, dams were injected with 2 mg/kg body weight Torbugesic (10 mg/ml butorphanol, Scan Vet Animal Health, Fredensborg, Denmark) subcutaneously to achieve analgesia. Anaesthesia was achieved by inhalation of isoflurane (Isoba Vet 100%, Intervet International, Boxmeer, The Netherlands). After the disappearance of voluntary reflexes (interdigital and skin-pinch), caesarean was performed by laparotomy through linea alba exposing the uterus. Excision of fetuses was done one at a time starting from the apex of the left horn towards the basis and subsequently commencing at the apex of the right horn. Immediately following delivery of each pup, the body weight was recorded, an intracardial blood sample was taken and the pup was euthanized by decapitation, the procedure lasting no more than 2 minutes. In the event of a pup displaying reflexes, euthanasia by intraperitoneal injection of 0.5 ml pentobarbital (200 mg/ml) supplemented with lidocaine (Veterinary Pharmacy, University of Copenhagen, Denmark) was performed. Gender was recorded and post mortem autopsy with tissue sampling was performed on each pup, tissues allocated either to fixative or frozen for later analysis. Blood samples were centrifuged, stabilized and frozen after the intracardial blood sampling of the final pup from each dam. Once all the pups were removed from the uterus, thoracotomy of the dam was performed and an intracardial blood sample was taken before sacrificing by decapitation and exsanguination.

The brain was removed and the left hemisphere frozen in liquid nitrogen and the right hemisphere fixated in 4% PFA (paraformaldehyde in phosphate buffered saline, 0.15 M, pH 7.5) for 48 h then transferred to 1%PFA for long term storage.

All frozen tissues were stored at –80 °C until further analysis. For the current study all foetal left brain hemispheres from a total of 85 fetuses, were blocked for gender and body weight and randomized to be used for gene and protein expression analysis (*N* values; 12 CTRL/GD 45, 9 DEF/GD 45, 10 CTRL/GD 56, 11 DEF/GD 56) or biochemistry (*N* Values; 12 CTRL/GD 45, 10 DEF/GD 45, 10 CTRL/GD 56, 11 DEF/GD 56).

Biochemistry

Analysis of VitC and malondialdehyde (MDA) in brain were performed as described previously [28,29,31]. Briefly, tissue samples (app. 0.5 g) were homogenized in PBS, centrifuged at 16,000 × *g* for 1 min at 4 °C. For VitC analysis, an aliquot was stabilized with an equal volume of 10% meta-phosphoric acid containing 2 mM EDTA (Merck, Whitehouse Station, NJ, USA), centrifuged, and the supernatant analysed by high-performance liquid chromatography (HPLC) with colorimetric detection. Levels of MDA were assessed by thiobarbituric acid derivatization followed by specific quantification of the genuine MDA(TBA)₂ adduct by HPLC with fluorescence detection.

Analysis of α-tocopherol and γ-tocopherol was performed by HPLC with coulometric detection as modified from Sattler et al. [46]. Briefly, to 100 µl of tissue homogenate was added 25 µl freshly prepared 2,6-di-*tert*-butyl-*p*-cresol (10 mg/ml; Sigma, Copenhagen, Denmark), 100 µl sodium dodecyl sulphate (29 mg/ml, Sigma), 800 µl H₂O, 900 µl ethanol and 100 µl 2-propanol. The cold mixture was extracted with 1 ml of *n*-hexane (Merck, Darmstadt, Germany) of which 500 µl of organic phase was reduced to dryness at 40 °C using an airstream and subsequently redissolved in 100 µl ethanol for 2 min using a vortex mixer. Following centrifugation, 20 µl of the supernatant was used for HPLC analysis. Superoxide dismutase activity (SOD) was analysed using the Ransod colorimetric assay (SD125, Randox Laboratories Limited, UK) on tissue lysates according to manufacturer's instructions.

RNA extraction and RT-PCR

Approximately 25 mg of each of brain tissues was homogenized in trizol (Invitrogen, Merelbeke, Belgium) and precipitated with chloroform (Sigma Aldrich, Steinheim, Germany) and isopropanol (Merck, Darmstadt, Germany). Purified RNA (SV Total RNA Isolation System, Promega, Madison, WI, USA) was eluted with 50 µl nuclease free water and the purity of RNA was determined by absorbance ratios A260/A280 and A260/A230 (Nanodrop 2000; Thermo Scientific, Wilmington, DE, USA). cDNA synthesis was performed by RT-PCR with 2 µg of RNA (MmLV RT enzyme, 5 × MmLV buffer and RNasin (Promega)); 10 mM dNTPs and Oligo (dT) primers (60 µg/120 µl) (Fermentas GmbH, St Leon Roth, Germany); Random hexamer primer (2 µg/µl) (GE Healthcare, Uppsala, Sweden).

Gene expression analysis

Intron-spanning beta-actin primers were used on all cDNA (Table 1) prior to real time quantitative PCR (Q-PCR) to test for genomic contamination. None of the included samples displayed signs of contamination. PCR products of included genes were run on 2% agarose gels to confirm the product size and were then purified by PCR clean-up (PCR Clean Up System; Promega, Sweden) and subsequently submitted for sequencing (LGC genomics, Berlin, Germany) to confirm specificity.

Q-PCR was conducted (SYBR Green I master LC480 and LC480, Roche, Basel, Switzerland) in 96-well white plates (Roche, Mannheim, Germany) with all samples in triplicates (in dilution 1:5), nuclease free water as negative control and calibrator as positive control. Gene expression analysis of *Caspase 3* from brain samples was normalized to the reference gene, *s18* (ribosomal protein S18). Primers are listed in Table 1.

Protein extraction and Western blot

Protein was extracted from brain tissue with radio-immuno-precipitation assay buffer (RIPA: 150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris, pH 8) with 1:100 protease inhibitors (Sigma complete protease inhibitor cocktail). Estimation of protein was done by bicinchoninic acid assay (Calbiochem Novagen BCA Protein Assay kit), according to the manufacturer's protocol, in triplicates at 562 nm on a SpectraMax Plus 384 UV/VIS plate reader (Molecular Devices Inc., CA, USA).

Approximately 100 µg of protein diluted with reducing agent and loading buffers (Invitrogen NuPAGE 4X LDS sample buffer, Invitrogen NuPAGE 10X sample reducing agent) were heated at 70 °C for 10 min before loading on precast gels (Invitrogen NuPAGE 4–12% Bis-Tris gels) in duplicates together with an internal standard comprising equal volumes of all samples. Gel to PVDF membrane transfer of protein was performed in a semi dry transfer unit (TE 77PWR, Amersham Biosciences) prior to 1 h

blocking in 2% blocking buffer (Amersham ECL Prime Blocking Agent in PBS-T wash buffer, 1XPBS and 0.1% Tween) before incubation with cleaved anti-caspase-3 (9661, Cell Signaling Technology, Danvers, MA, USA) or 4% blocking buffer before incubation with anti-nitrotyrosine (anti-NT, 06-284, Millipore, Temecula, CA) or anti-4-hydroxynonenal (anti-HNE, HNE 11-S, Alpha Diagnostic International, San Antonio, TX, USA). Antibody specificity of cleaved caspase-3 was tested by pre-absorption with equal volume of the blocking peptide (#1050, Cell Signaling Technology, Danvers, MA, USA) and specificity of anti-nitrotyrosine was tested by pre-absorption of 0.5 µg/ml primary antibody with 15 mM free NT (#89540, Cayman Chemical, Ann Arbor, MI, USA) with respective controls (#9663 Caspase-3 control Cell signalling technology, Danver, MA, USA); (#12–354, NT immunoblotting control, Chemicon, Temecula, CA, USA). Membranes were incubated with primary antibody overnight at 4 °C (1:1000, cleaved caspase-3 or 0.5 µg/ml anti-NT or 1:2000 anti-HNE), followed by secondary antibody (anti-rabbit IgG-HRP, 7074, Cell Signaling Technology, Danvers, MA, USA) 1: 1000, 1:6000 and 1:2000 for anti-caspase-3, anti-NT and anti-HNE proteins respectively. Imaging was achieved by enhanced chemiluminescence (Amersham ECL Prime Western Blotting Reagent, UVP Biospectrum imaging system). Band-sizes were identified by Western blot protein standard (Magic Mark™ XP Western Protein Standard). Cleaved caspase-3 bands were located by an additional protein standard (ECL DualVue Western Blotting Markers, RPN810, Amersham Biosciences) with a specific secondary antibody (1:10000, HRP-S) diluted together with the secondary antibody. The NT and HNE blots were stripped with 0.5 M sodium hydroxide (Merck, Darmstadt, Germany) before re-probing with loading control (mouse anti-actin, 1:20000, MAB 1501, Millipore, Temecula, CA, USA). Target bands of cleaved caspase-3 or lanes in case of NT and HNE blots were normalized to the loading control, detected by anti-mouse IgG-HRP secondary antibody (1:10000, SC-2005, Santa Cruz Biotechnology, CA, USA). UVP Life Science Series Image Acquisition and Analysis software was used for densitometry and blots were normalized to an internal control.

Statistics

SAS/JMP statistical version 8.0 was used to analyse all data. Analysis of Q-PCR expression data was performed by Student's *t*-test. Two-way ANOVA was used to analyse biochemistry and Western blot densitometry. Interaction between factors, diet and GD, was reported together with main effects when statistically significant ($p < 0.05$) followed by Tukeys HSD posthoc comparisons.

Results

Brain antioxidant status

As shown in Table 2, VitC concentration in the brain was significantly higher in the CTRL groups of both gestational days compared to DEF counterparts ($p < 0.0001$). Two-way ANOVA showed effects of both diet and GD ($p < 0.0001$) on VitC levels as well as an interaction between diet and GD ($p < 0.0001$). A decrease of approximately 20% in VitC was observed in CTRL GD 56 relative to GD 45 ($p < 0.0001$). This was not found in the DEF group, where VitC levels did not differ between gestational dates. In contrast to total VitC in the brain, α -tocopherol levels were high on GD 56 relative to GD 45 ($p < 0.0001$) with no significant effect of diet. Conversely, two-way ANOVA showed a significant effect of diet on γ -tocopherol ($p < 0.05$), the DEF-group displaying overall increased levels. However, tocopherols were not significantly different between CTRL and

Table 1
Primer sequences applied in the gene expression analysis.

Gene	Primer sequence	Product size (bp)	NCBI accession no.
Beta-actin	(F): gtaaggacctctatgcccaacaca	346	AF508792
	(R): atgcaatctctatctgttttct		
s18	(F): atgtggtgttgaggagaagcag	195	XM_003473925.1
	(R): gcttggtgtccagaccgttg		
Caspase-3	(F): ctggaatggcagctcgactt	315	XM_003469171.1
	(R): cccggcgaagcctgaataat		

Sequences are presented in the 5'–3' direction (F): forward, (R): reverse.

Table 2
Brain antioxidant status in prenatally VitC deficient and control guinea pigs at GD 45 and GD 56.

	CTRL GD 45	CTRL GD 56	DEF GD 45	DEF GD 56	Effect of VitC	Effect of GD
VitC (nmol/g tissue)	3043 ± 285 ^a	2351 ± 137 ^b	1016 ± 270 ^c	966 ± 242 ^c	***	***
α-Tocopherol (nmol/g tissue)	5.5 ± 0.8 ^b	9.0 ± 1.0 ^a	4.6 ± 1.0 ^b	9.4 ± 1.5 ^a	NS	***
γ-Tocopherol (nmol/g tissue)	0.52 ± 0.46	0.50 ± 0.36	0.80 ± 0.5	0.89 ± 0.39	*	NS
Superoxide dismutase (U/g tissue)	90 ± 2 ^a	92 ± 2 ^a	133 ± 3 ^b	137 ± 4 ^b	***	NS

CTRL: controls; DEF: deficient. GD: gestational day. Values are presented as means ± SD. Differences between groups were assessed using two-way ANOVA with diet and GD as factors followed by Tukeys HSD test for individual comparisons. NS: not significant. Different superscript letters indicate that groups are significantly different.

**p* < 0.05.

***p* < 0.001.

****p* < 0.0001.

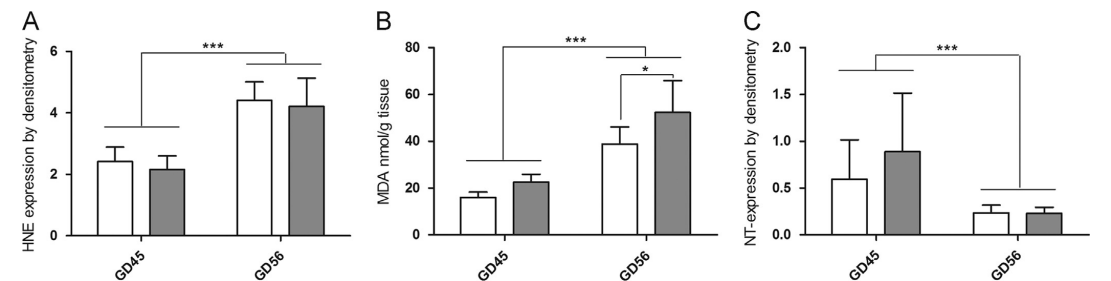


Fig. 1. Oxidative damage in foetal guinea pig brains. Markers of increased oxidation and suggestive oxidative damage in the brain of guinea pigs subjected to prenatal VitC deficiency (DEF) (dark bars) or control diet (CTRL) (light bars) during development. Foetuses were excised by caesarean section at GD 45 and GD 56. (A) Densitometric analysis of HNE modified proteins in brain homogenate; results are normalized to actin; (B) Malondialdehyde in brain homogenate, measured by HPLC; (C). Densitometric analysis of nitrotyrosine modified proteins normalized to actin. Data are expressed as means ± SD. **p* < 0.05; ****p* < 0.0001 by ANOVA.

DEF groups at the individual time-points. SOD was significantly increased by VitC deficiency (*p* < 0.0001) but was not affected by GD.

Brain oxidative damage

Western blot of HNE modified proteins ranged between 20 and 120 kDa in guinea pig brain lysates (data not shown). Two-way ANOVA of densitometry data showed that the intensity of HNE modifications at GD 56 was twice as high as GD 45 with a significant effect of GD (*p* < 0.0001) (Fig. 1A). However, no significant differences were found between CTRL and DEF groups at either gestational day. MDA was measured in brain homogenate as a marker of lipid oxidation. Two-way ANOVA showed significant effects of both diet (*p* < 0.001) and gestational age (*p* < 0.0001). In agreement with the anti-HNE densitometry data, MDA was significantly elevated at GD 56 compared to 45 for both dietary regimens separately (*p* < 0.0001). Moreover, VitC deficiency resulted in increased brain MDA compared to controls at GD 56 (*p* < 0.05) but at GD45, the increase did not reach statistical significance (Fig. 1B).

NT modified proteins were detected by Western blotting between 35 and 15 kDa in brain lysates. Pre-absorption of primary antibody with free NT abolished all the bands in guinea pig brain lysate and almost all bands in the positive control confirming specificity of the antibody (data not shown). A significant main effect of GD (*p* < 0.0001) was detected with highest levels of NT modified proteins in GD 45 brains (Fig. 1C). There was no detectable effect of diet on the level of NT modified proteins at either gestational day.

Caspase-3 expression

Q-PCR analysis of *caspase-3* gene-expression in the brain was not significantly different between CTRL and DEF groups in

either gestational day (Fig. 2A). Cleaved caspase-3 Western blotting showed a single band just below 15 kDa. Pre-absorption of primary antibody with blocking peptide abolished the specific bands from guinea pig brain lysate and the positive control confirming the specificity of the antibody (data not shown). Two-way ANOVA of densitometry data showed that GD had a significant effect on cleaved caspase-3 (*p* < 0.05) with higher levels at GD 45 compared to GD 56 (Fig. 2B). Densitometry analysis at either gestational day did not show significant differences between CTRL and DEF groups (Fig. 2B).

Discussion

In the present study, we show that immature brains are susceptible to oxidative stress in spite of an induction of protective adaptation mechanisms. Oxidative stress and damage markers in guinea pig cerebral cortex were assessed at preterm and near term gestational time points with and without maternal VitC deficiency as a potential oxidative insult.

Brain VitC levels in DEF groups were significantly lower than CTRL as expected, and while VitC levels in the DEF group remained similar at the two gestational days, VitC in GD 56 CTRL was significantly lower than GD 45 CTRL. This finding indicates a developmental requirement of increased VitC during the preterm period possibly as a response to lower protective mechanisms against oxidizing agents as has previously been suggested [36]. However, in contrast to CTRLs, the DEF group did not display a near-term drop in concentration but rather showed retention of VitC, possibly to avoid any further loss than the already existing low level. The decrease in VitC toward term in CTRL animals is in line with findings in humans reporting a late gestation decrease of VitC in all foetal tissues including the brain [58,59]. This decrease in the brain has been suggested to be due to growth

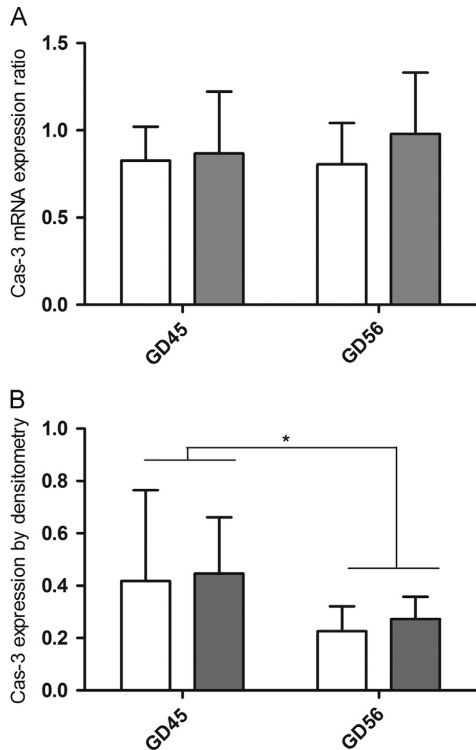


Fig. 2. Expression analysis of caspase-3 in the brain. (A) Quantitative PCR analysis of *caspase-3* in guinea pig foetal brains comparing vitC deficient (dark bars) to controls (light bars) on two gestational time points (GD45 and 56). Expressed values are normalized to the reference gene *s18* and displayed as mean \pm SD. (B) Densitometric analysis of levels of cleaved caspase-3 obtained by Western blot and normalized to actin levels. Data are expressed as means \pm SD. * $p < 0.05$ by ANOVA.

and maturation of non-neuronal cells and maturation and myelination of neurones [16,59]. Recent studies by us have shown that a pre-natal low VitC causes a reduction in foetal body and brain weight at GD 45 but not at GD 56 (unpublished results), indicating that a developing foetus is able to compensate for a pre-imposed negative effect of VitC deficiency during the final weeks of gestation.

Our α -tocopherol data implies that it increases with progression in development during late gestation. Interaction between cytosolic VitC and membrane bound vitamin E regenerates oxidized vitamin E and results in an efficient antioxidant mechanism [9], which suggests that VitC deficiency may alter vitamin E levels hereby possibly disrupting membrane integrity. Some in-vivo studies have shown that intake of high VitC results in an increased vitamin E in tissues [5,17,18]. This was not the case when evaluating α -tocopherol in CTRL vs. DEF groups of this study, however, VitC showed a main effect on γ -tocopherol levels in the brain. γ -Tocopherol has been associated with anti-inflammatory properties, decreased superoxide anion generation and inhibition of lipid peroxidation, hereby reducing adverse effects of oxidative imbalance [23,45]. Our present findings suggest an increase in γ -tocopherol in response to VitC deficiency, whereas levels during late gestational progression were relatively constant in both CTRL and DEF groups.

In the current study, VitC deficiency increased SOD activity during late gestation. SOD activity in CTRL groups at both GD is in

agreement with a previous report of constant SOD levels in guinea pig foetal brains throughout gestation from GD 30 to GD 60 [36]. As a consequence of low VitC levels, increases in SOD activity in DEF compared to CTRLs at both GD indicates foetal ability to induce a compensatory mechanism, likely due to increased production of superoxide radicals [24]. Various studies have reported an increase in SOD activity in the brain as an adaptive mechanism to tolerate oxidative damage in neurodegenerative conditions [41], stress [10] and oxidant induced neurotoxicity [6]. Studies like these with additional assessment of antioxidants like catalase, glutathione peroxidase or oxidative damage markers helps in understanding whether such adaptive mechanisms are able to preserve the redox status [3,57].

HNE modified proteins, which indicate oxidative damage due to lipid peroxidation, were significantly high at GD 56, regardless of VitC status, likely due to the increase in poly-unsaturated fatty acids in the brain at near term. An increased susceptibility of lipid peroxidation in near term brains of guinea pigs has previously been reported [37]. Although HNE modified proteins were not significantly different between CTRL and DEF, a trend similar to the obtained MDA levels was observed between the two GD, with significantly higher MDA levels at near term further supporting the maturational related increase of lipid peroxidation in the brain as reported by others in guinea pigs brain [37]. Significant increase of MDA levels in DEF at GD 56 and not in DEF at GD45 compared to their CTRL suggests that compensatory mechanisms that may have been present at earlier gestation may not be enough to protect from lipid peroxidation due to low levels of VitC in the brain. Earlier studies by us and others have shown a similar inverse relation of VitC levels to lipid peroxidation in the brain [15,33] and the vital role of ascorbate has previously been demonstrated to inhibit lipid peroxidation in rat brain microsomes [47]. Conversely, no significant differences in NT modified proteins between CTRL and DEF suggests that posttranslational modification of proteins by NT is not effected by VitC deficiency and instead, as proposed by others, NT may be involved in mediating cell signalling at its basal levels during development [27,35].

Results from oxidative stress based in-vivo studies have suggested an increased free radical production or decreased antioxidant mechanisms in the immature brain [36,56], which in turn may initiate cellular events to initiate mechanisms of apoptosis [8,42] like caspase-3 activation [22]. Our finding of high levels of cleaved caspase-3 in GD 45 compared to GD 56 indicates a developmental phenomenon in agreement with what has been shown by others [2], placing cleaved caspase-3 as playing an important role in reducing neuronal overproduction in the developing brain [54]. The guinea pig is a precocial species and neuronal number relative to adult stage are achieved by GD 48 [12]. Hence, although a gestational effect was seen for cleaved caspase-3, the difference was small implying that most of the cell death associated with development is likely to have occurred before the investigated time-points. No significant differences in *caspase-3* gene expression or cleaved caspase-3 protein levels between CTRL and DEF groups at any of the GDs indicates that VitC deficiency does not modulate caspase-3 mediated apoptosis and may not be the underlying mechanism associated with hippocampal impairment observed in our earlier findings [51,52].

Conclusion

Guinea pig foetal brains have differential requirements of antioxidants with high VitC levels at pre-term, a higher requirement of α -tocopherol at near term and constant SOD activity. Oxidative NT modifications and active caspase-3 levels are higher at pre-term and are prone to increased lipid peroxidation at near

term signifying differential expression of oxidative damage markers associated with brain maturity. Prenatal VitC deficiency in the guinea pig foetal brain does not modulate levels of vitamin E, NT or HNE protein modifications or caspase-3 however, increases SOD activity as compensation although this is not adequate to prevent increased lipid peroxidation at the investigated time points.

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